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EFFECTS OF BORON ON THE WATER RELATIONS OF HIGHER PLANTS[1,2]

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Schmucker (20) demonstrated that the presence of 0.001 to 0.01 % boron (as boric acid) in the solution, in which pollen grains of Nymphaea were germinating, prevented the rupture of pollen grains and tubes and also stimulated growth of the tubes. He attributed this beneficial effect of boron to the fact that it regulated the hydration of colloids. Schmucker stated that boron may be associated with polyhydroxyl compounds of the membrane and that boron may have an effect on the formation of pectic substances. Dennis (4), Minarik and Shive (18), and Schropp, according to Thomas (23), also suggested that boron may decrease the hydrophilic capacity of the plasma colloids.

Minarik and Shive (18) reported an inverse relationship between the percent of moisture in soybean leaves and the level of B in the substrate. They concluded that their results substantiated Schmucker's hypothesis that the degree of hydration of cells is regulated by B. A number of investigators have failed to confirm this relationship (2, 6, 17, 19, 21). The positive relationship, which these latter authors observed, does not necessarily discredit Schmucker's explanation as to why pollen grains burst in a solution lacking B. It will be shown later that the measurement of percentage moisture in B-deficient and normal (B-sufficient) plant parts is not necessarily a measure of the relative capacities of leaf tissues to hydrate.

In addition to a proposed role of boron in the formation of cell walls and membranes, it has been shown that the transpiration rate is higher in normal as compared to B-deficient plants (5, 15). Furthermore, Dorfmüller (5) found that the reverse of this is true if the leaves are excised before their water-loss is measured. However, he did not completely describe the phenomenon in that he did not thoroughly study the initial water loss of excised leaves.

In view of the conflicting reports concerning the effect of boron on water relations, a number of experiments were conducted in an attempt to determine the direction of response and, if possible, to elucidate the mechanism of action.

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MATERIALS AND METHODS

An automatically-irrigated sand culture system (11) was used to produce the experimental plants. Each 5-gallon crock culture received 0.5 gallon of solution hourly except between 8 P.M. and 6 A.M.

Separate molar solutions of $Ca(NO_3)_2$, KH_2PO_4, and $MgSO_4$ supplied the macro-elements. The trace elements were supplied by 3 stock solutions, viz., H_3BO_3, monosodium, iron, ethylenediaminetetraacetate, and a "supplementary solution" (consisting of $CuSO_4$, $ZnSO_4$, and $MnCl_2$). Demineralized water was used to make the stock and nutrient solutions. The composition of the nutrient solution was as follows:

MEQ/L						PPM				
Ca	Mg	K	NO_3	H_2PO_4	SO_4	Cu	Zn	Mn	Fe	B
10	4	4	10	4	4	0.02	0.05	0.5	3	Varied

The solutions were changed every 10 days to 2 weeks.

Snap bean, *Phaseolus vulgaris* L. cv Black Valentine, sunflower, *Helianthus annuus* L. cv Russian Mammoth, and tomato, *Lycopersicon esculentum* Mill. cv Marglobe, were used in these experiments.

For the water-loss experiments, usually the leaves of normal and B-deficient plants were excised, the petioles were immersed in water, and the leaves were then carried from the greenhouse to the room where the balances were located. An alternate method involved placing the leaves in polyethylene bags for transport to the balances. A leaf from a normal plant and one from a B-deficient plant were hung on the beams of separate analytical balances at the same temperature and under similar lighting conditions.

Measurements were also made of the transpiration from attached bean leaves of 7-weeks-old plants, which were beginning to fruit, using the method originally devised by Freeman (7). Glass cups were sealed to the lower surfaces of the leaves by a latex product (Loxite 2109) supplied by the Firestone Rubber Company. A flow of air through the cup was effected by a slow rate of flow of water (8 gals/hr) from an aspirator bottle and the transpired water was collected in a pair of $CaCl_2$ drying tubes. Two control tubes were also used in each experiment to measure

the amount of water in the same volume of atmosphere so that the appropriate correction could be made. Measurements were made between 11 A.M. and 3 P.M. on clear days with the temperature in the greenhouse ranging from 25 to 30° C.

Using expressed sap of leaves from plants subjected to the two treatments, osmotic pressure determinations were made using a Beckman cryoscopic apparatus (12, 24). Leaves of one-month-old bean plants were used in the first experiment and leaves of 6-weeks-old plants in the second one. The electrical conductivity of the plant sap was determined with a conductivity bridge, using a pipette form of electrode. By dividing the conductance values (in $K \times 10^5$, 25° C) by 250, an approximation could be made (26) of the fractions of the total atmospheres osmotic pressure contributed by electrolytes.

A polysaccharide fraction, which consisted of pentosans and pectic substances, was measured by the official method of the Association of Official Agricultural Chemists (16). Leaves of 7-weeks-old bean plants were harvested, diced, and thoroughly mixed to insure a homogeneous sample. They were taken from the same position on 10 plants in each treatment. The harvesting of leaves of 5-weeks-old sunflower plants was carried out in a similar manner, the leaves being taken from the top 4 nodes. The plant material was heated with 12 % HCl in an all-glass-distilling apparatus. The distillate of furfural, produced from pentosans and pectic substances, was precipitated as furfural phloroglucide with phloroglucinol and then weighed in tared Gooch crucibles with asbestos mats.

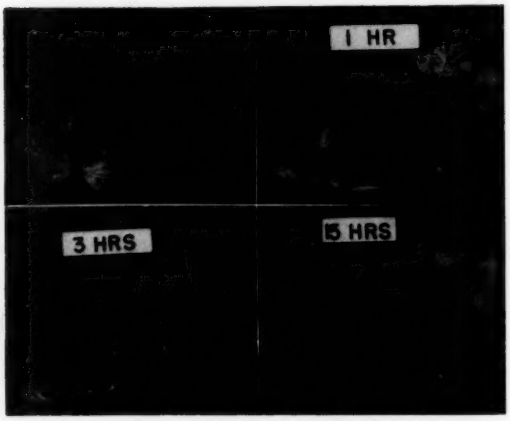

FIG. 1. Comparative wilting, from the time of cutting (*upper, left*) through a period of 15 hrs, of an excised leaf from a B-deficient bean plant (*right*) and a leaf from a plant supplied with B (*left*).

As a measure of the relative drying rates of the two types of tissue, leaves of B-deficient plants expressing marked deficiency symptoms and B-sufficient plants were dried at 75° C and ground to pass a 40-mesh sieve. Two samples each of normal and B-deficient leaf tissue were placed on asbestos pads in Gooch crucibles and arranged randomly in filter flasks. Fifteen ml H_2O was added to the samples and time was allowed for the plant material to imbibe water. A common vacuum of 0.7 atm of tension was then applied, and suction was discontinued when water ceased to come through. The crucibles and contents were then reweighed.

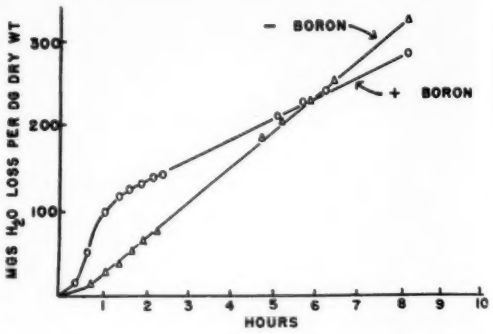

FIG. 2. Accumulative water loss of excised bean leaves.

In the study of epidermal and stomatal modification, portions of the lower epidermis were separated from the rest of the leaf. These areas of epidermis were then observed microscopically. In the preparation of slides for anatomical study, leaf segments were fixed with formalin-acetic acid-alcohol (13) and dehydrated by the tertiary-butyl alcohol method. The sections were stained by safranin and fast green.

EXPERIMENTAL RESULTS

Excised leaves of B-deficient plants were slower in wilting and drying than the corresponding leaves of normal plants (fig 1), as has also been reported by Leaf (15). When water loss was measured against time it was observed that the leaves of B-deficient bean and tomato plants lost water linearly with time, but that the leaves of the normal plants lost water very rapidly immediately after they were excised (figs 2 and 3). The normal leaves attained rates of water loss which were linear with time after the first hour. The slopes of the curves show that the rates of water

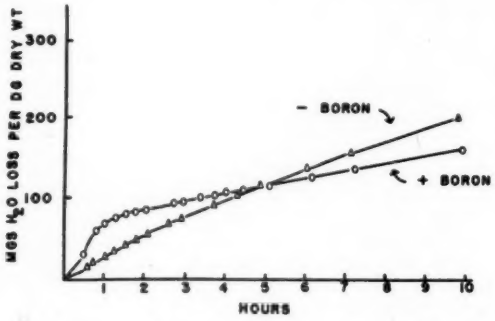

FIG. 3. Accumulative water loss of excised tomato leaves.

TABLE I

TRANSPIRATION * OF INTACT LEAVES OF NORMAL AND BORON-DEFICIENT BEAN PLANTS

TREATMENT	OBSERVATION										MEAN
	1	2	3	4	5	6	7	8	9	10	
−B	66	14	70	81	47	25	45	67	61	62	54.0
+B	100	81	136	180	230	282	194	249	160	174	179.0
L.S.D., 1%	...	..	...	...	...	...	...	...	...	...	70.0

* Measured in mg H_2O per 7.1 cm^2 leaf area per hr.

loss from the normal leaves were less than that of the B-deficient leaves after the first hour. Dorfmüller (5) made a similar observation but failed to characterize the initial water loss of excised leaves. The moisture percentages of leaves from the two treatments were not very different, i.e., for the bean leaves the normal one contained 89.5 % water while the deficient one contained 87.7 % water. The normal tomato leaf contained 86.2 % water and the deficient leaf contained 88.9 % water.

Dorfmüller observed that the transpiration rates of intact normal plants were higher than those of B-deficient plants. According to Leaf (15), Biebl also observed this relationship. This finding was corroborated with a leaf-cup apparatus (table I). This pattern of behavior is opposite to that of the excised leaves after the first hour (figs 2 and 3).

In an attempt to elucidate the phenomena with excised leaves, the osmotic pressure and electrical conductivity of expressed sap of normal and B-deficient bean leaves were determined. Inasmuch as the total

TABLE II

OSMOTIC PRESSURE, ELECTROLYTE, AND CALCULATED NON-ELECTROLYTE VALUES OF LEAVES OF MATURE BEAN PLANTS *

EXPERIMENT	SAMPLE	ATMS OSMOTIC PRESSURE		
		ELECTROLYTE CONTENT	NON-ELECTROLYTE CONTENT	TOTAL OSMOTIC PRESSURE
I	+B			
	Top leaves	5.9	6.0	11.9
	−B			
	Top leaves	5.7	6.9	12.6
II	+B			
	Top leaves	5.5	2.7	8.2
	Middle leaves	6.1	2.5	8.6
	Lower leaves	4.9	2.3	7.2
	−B			
	Top leaves	6.1	4.5	10.6
	Middle leaves	5.8	3.8	9.6
	Lower leaves	5.3	3.7	9.0

* All values are the means of duplicate samples. Individual measurements in experiment I varied from the mean by less than 3 %, and in experiment II by less than 6 %.

TABLE III

THE RETENTION OF WATER BY GROUND (40-MESH), WETTED BEAN LEAF TISSUE UNDER 0.7 ATM OF TENSION

SAMPLE	DRY WT OF SAMPLE	GMS H_2O HELD/GM DRY WT OF SAMPLE	AV. GMS H_2O HELD/GM DRY WT
+B (a)	2.573	1.465	1.577
(b)	2.998	1.689	
−B (a)	2.502	2.679	2.572
(b)	2.817	2.464	

osmotic pressure was higher in B-deficient than in normal leaves—and the electrolyte concentrations were similar—the B-deficient plants were found to have a higher concentration of non-electrolytes, commonly assumed to be "sugars," than normal plants (table II). The higher concentration of sugar in B-deficient plant leaves corroborates the findings of other investigators (9, 14). Similar results were obtained for the leaves of tomato plants, the B-deficient leaves having a total osmotic pressure of 8.5 atms as compared to 4.4 atms for the normal leaves.

An experiment was conducted to determine whether ground (40-mesh), wetted tissue of normal and B-deficient bean plants differed in ability to retain water against a partial vacuum. It was found that the quantity of water present in the B-deficient leaf tissue, after 0.7 atm of tension was applied, was about 64 % greater than that of the normal leaf tissue (table III). In order to determine the nature of the "hydrophilic colloids" contributing to the increased power of hydration of B-deficient tissue, pentosans and pectic substances were measured first as one group. It was found that this group of hydrophilic substances is present in a higher concentration in the

TABLE IV

FURFURAL-YIELDING SUBSTANCES (PENTOSANS AND PECTINS) IN THE LEAVES OF BORON-DEFICIENT AND NORMAL BEAN PLANTS

SAMPLE	MGS FURFURAL PHLORO-GLUCIDE/10 GM FRESH WT	CALCULATED MGS FURFURAL/10 GM FRESH WT	% INCREASE OVER NORMAL
	Top leaves		
B-deficient			
(a)	125	74	54
(b)	123	73	
Normal			
(a)	81	48	
(b)	80	48	
	Middle and lower leaves		
B-deficient			
(a)	93	55	19
(b)	95	57	
Normal			
(a)	84	50	
(b)	74	44	

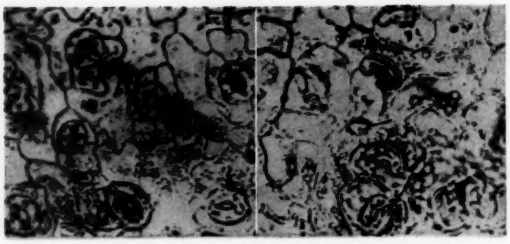

FIG. 4. Photomicrographs of the epidermis showing abnormal stomates on a boron-deficient bean leaf (*right*) as compared to normal ones on a normal leaf (*left*).

B-deficient leaves of bean plants (table IV). Similar data were obtained with sunflower leaf tissue. Inasmuch as the moisture percentages of the leaves differed by only 2 or 3 %, it is possible to express the phloroglucide values of both types of leaves on a fresh weight basis.

Observations on the epidermal layers showed marked differences between the leaves of normal and B-deficient bean leaves (fig 4). The epidermal cells of the B-deficient leaves were distorted and there were many non-functional stomates. Normal cell expansion appeared to be seriously impeded in the epidermal layer of B-deficient leaves. Many investigators have reported that B-deficiency deleteriously affects cell division. (10, 22). Cross sections of leaves showed a deranged condition in deficient plants in which irregular growth produced thickened spots in the leaves (fig 5). The B-deficient leaves were also actually thicker than normal leaves on the average.

DISCUSSION

It was observed that there is a marked difference in water loss from excised normal and B-deficient leaves. After the first 20 min, the normal bean leaf had lost 6.0 % of the total water present in the leaf as compared to 2.0% by the B-deficient leaf. This first measurement of water loss should approximate the relative transpiration picture for intact plants. Different forces are involved in the retention of the water that is lost soon after the excision of leaves as compared with the water that is lost later on.

The osmotic pressure of top leaves of B-deficient plants averaged 1.5 atm above that of the normal leaves. Most of this difference could be attributed to

FIG. 5. Photomicrographs of comparable cross-sections of leaves of boron-deficient (*right*) and normal (*left*) bean plants.

the difference (about 0.05 M, expressed as sucrose) in the concentration of "sugars" (24). Such a difference would not decrease the vapor pressure of the cell solution to a very great extent since 1 molal solutions of sucrose and glucose have vapor pressures only 1.97 % and 1.72 %, respectively, lower than that of pure water (27). However, in view of Boon-Long's experiments (3), this factor should not be disregarded entirely in a study of water loss from normal and B-deficient leaves. His data show a 40 % decrease in transpiration of normal excised bean leaves which had been allowed to photosynthesize for 2 days as compared to similar leaves kept in the dark. The petioles of leaves receiving light had been scalded to prevent an outward movement of sugars. After the leaves were excised, the petioles were sealed in 10-ml vials filled with water and weighing was then begun. Freezing point determinations of the saps of these leaves showed that the vapor pressure of the sap of leaves receiving light was decreased only 0.3 % as compared to the leaves kept in the dark. He concluded that this reduction in transpiration was due to the fact that sugar decreased the permeability of the membranes to water.

It is postulated here that the longer time required, for a given degree of wilting of B-deficient leaves, was not entirely due to an increased sugar concentration in the leaves. The higher concentration of "pentosans and pectic substances," which was also observed, would increase the hydrophilic capacity, increase the retention of water, and result in a relatively longer period being required for complete wilting. Ashworth (1) showed that there is more pectin in B-deficient sunflower tissues, and Johnston and Dore (14) obtained similar results with tomato plants. This relation was corroborated by data presented here. It is suggested that this increase in polysaccharides may be due to the presence of an increased amount of precursor compounds in the B-deficient leaf which can be converted to these polysaccharides. A higher proportion of water in the B-deficient leaf could thus be associated with these substances and this would explain why leaves of normal and B-deficient plants can have practically the same moisture percentage and yet the B-deficient ones contain more hydrated colloids.

The decreased rate of transpiration of attached leaves of B-deficient plants, reported herein and by other investigators (5, 15), is postulated to be due to at least three factors. First, indirectly by the measurement of water uptake (15), the absorption rate of water has been shown to be decreased in B-deficient tobacco plants. This is probably associated with a diseased, very much reduced root system, which has been observed in this and other work. Second, there is clearly a higher sugar and colloid concentration in leaves of B-deficient plants. This factor could influence the evaporation rate when a differentially permeable membrane is involved. Third, there is a reduced number of functional stomates. There is a derangement in cell size and configuration of various cells, in addition to that of the guard cells, in B-deficient

leaves. The way that B-deficiency brings about these effects is not known at the present time. Owing to death of the terminal meristem, a deficiency of auxin would be expected to occur in leaves of B-deficient plants. Frey-Wyssling (8) stated that auxin may exert its effect on cell expansion through activation of enzymes which degrade cellulose in localized areas. Van Overbeek (25), on the other hand, stated that auxin appears to exert its effect on cell expansion by stimulating pectin-methylesterase which initiates the breakdown of the water-insoluble pectin of the primary wall, thus decreasing its tensile strength and allowing it to stretch. Either mechanism could explain the distorted cell growth which was observed in B-deficient leaves. The abnormal stomata, composed of only one typical guard cell, would most certainly affect the rate of water loss from the leaves of B-deficient plants.

With regard to drought resistance, it was observed in the greenhouse that a sudden rise in temperature from 80 to 100° F resulted in extreme wilting of normal bean plants whereas the B-deficient plants showed no visible loss of turgor. These results are in contrast to the results obtained by Willis and Piland (28) in which they observed an increased wilting on hot days of Romaine lettuce plants which had not been fertilized with boron. It is difficult to evaluate this observation in view of the fact that there may have been factors which could not be assessed in a field experiment. Dorfmüller reported that B-deficient legumes were slower in decreasing their transpiration rates and becoming adapted to a reduced water supply. It is concluded from observations recorded herein, however, that B-deficient bean plants are more resistant to a sudden moisture stress. This conclusion can be supported by arguments similar to the ones used in explaining the differences in the transpiration and water loss of normal and B-deficient leaves, attached and excised, respectively.

Summary

The transpiration rate of B-deficient bean plants is much lower than that of normal plants. This reduction is due to at least 3 factors. First there is a higher sugar and colloid concentration in B-deficient leaves. Second, a decreased rate of water absorption has been reported for B-deficient plants. Third, transpiration is affected by an abnormal leaf morphology including a high percentage of non-functional stomates.

A relatively rapid initial water loss from normal excised leaves of bean and tomato plants, which gradually levels off into a linear rate as compared to a linear rate from the start with B-deficient leaves, is interpreted as being due to: (a) a higher concentration of sugar in B-deficient leaves, and (b) a higher proportion of the water in B-deficient leaves being associated with hydrophilic colloids, and (c) a reduced number of functional stomates on B-deficient leaves.

A higher concentration of pectins and possibly pentosans has been shown to occur in the leaves of B-deficient plants. This is believed to be a factor in the slower wilting of B-deficient plants under a moisture stress.

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STUDIES ON THE GROWTH OF COLEOPTILE AND FIRST INTERNODE SECTIONS. A NEW, SENSITIVE, STRAIGHT-GROWTH TEST FOR AUXINS[1,2]

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The demand for an improved bioassay of the substances which promote and inhibit growth in plants is apparent from the large number of tests which have been suggested in recent years and has prompted the efforts which will be reported in this paper. The present investigation resulted in (a) the improvement of the well-known coleoptile section test and (b) the working out of a new test using as a test organ the first internode of the oat seedling.

The qualities which one looks for in such tests are: sensitivity, large proportionality range between the concentration of the active substances and the growth response, specificity, reliability, and ease of performance. Let us examine critically the main auxin tests which are based on the measurement of cell elongation to see how well they meet these criteria. From the short and incomplete review summarized in table I it is apparent that the Avena curvature test is a good and sensitive test. It is not well suited, however, to the assay of paper chromatograms for three reasons: 1) the procedure is too delicate, requiring time and skill; 2) the proportionality range is too small; 3) the detection of inhibitors is difficult, although this insensitivity to inhibitors may be an advantage in certain cases. The pea curvature test, on the other hand, would appear to be suitable, but its lower limit of sensitivity is too high for usual paper chromatograms and it does not detect inhibitors readily. Many workers have resorted to the coleoptile straight growth test to detect auxins and inhibitors on paper chromatograms. This seems to be a simple test, requiring little apparatus. Unfortunately, the sensitivity of this test is much less than that of the Avena curvature test. Rietsema (28) reported the development of a sensitive coleoptile cylinder test, but, after six years, no one seems to have used this test, perhaps because of a lack of reproducibility. Roots, which are much more sensitive to auxins than shoots, have been used by several workers, for example Moewus (23), Audus and Thresh (1), etc. Unfortunately, roots generally respond to auxins by a growth inhibition, which makes them unsatisfactory to study growth promoting substances. Very low auxin concentrations, however, promote root elongation. The dome-shaped curve resulting from the succession of the promoting and the inhibiting effect, renders ambiguous the determination of the auxin concentration. Finally, it has been shown that the cress root test of Moewus is not very reliable for quantitative work, the limits of the error made at IAA concentrations below 1 μgm/l being from 50 to 260 % of the actual values (9). Also, the pea root test of Leopold and Guernsey (18) is very variable, to the point that it could not be repeated with a different batch of the same variety of peas (Leopold, private communication).

Screening of Various Test Objects: These observations led us to conclude that a straight growth test was the most desirable type for paper chromatography work and that one should look for a shoot material which would be more sensitive than the stem segments usually employed. Since roots appear to be more sensitive than shoots, we thought of using tissues which would be intermediary between shoots and roots, namely hypocotyls. However, a preliminary

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Part of this paper has been presented at the Conference on Plant Growth Substances held at Wye College, Kent, England, July 17 to 22, 1955 and can be found in the book entitled "The Chemistry and Mode of Action of Plant Growth Substances" published by Butterworths Publ. Ltd., 4 Bell Yard, London, W.C.2, England. Permission by the Butterworths company to publish this material is gratefully acknowledged.

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TABLE I

A COMPARISON OF SOME BIOASSAYS FOR AUXINS

TEST	SENSITIVITY, MINIMUM DETECTABLE IAA			USABLE RANGE *	RELATIVE ACCURACY **	SPECIFICITY	RELIABILITY	EASE OF PERFORMANCE, SPECIAL EQUIPMENT †
	CONC (μGM/L)	VOL (ML)	TOTAL AMT (μGM)	(μGM/L)				
A. Tests based on differential growth responses								
Avena curvature test (43)	5	0.3	1.5×10^{-3}	10 to 100	High	Very high	Good	Requires skill, controlled humidity, special small equipment.
Deseeded Avena test (31)	1	0.3	3×10^{-4}	2 to 100	High	High	Good	id
Split pea test (44, 38)	50	25	1.25	100 to 10,000	Medium	Low	Good	Requires little skill, little special equipment.
Quartered Avena test (40)	0.01	20	2×10^{-4}	0.3 to 10,000	Medium	?	Medium ?	id
B. Tests based on straight growth								
Avena section test (4, 7, 29)	10	10	0.1	10 to 3,000	Medium	Low	Good	Requires little skill, little special equipment.
Micro straight growth test (28)	0.01	2	2×10^{-5}	0.01 to 100	Medium	?	?	id
Pea section test (11, 39)	3	5	1.5×10^{-2}	3 to 300	Medium	Low ?	Good	Takes 7 days to grow the test plants. Requires little skill, little special equipment.
Cress root test (23)	0.001	10	1×10^{-5}	1 to 10,000	Low	Very low	Poor	Requires little skill, little special equipment.
Pea root section test (1)	0.001	0.75	7.5×10^{-7}	0.1 to 1,000	Medium	Low ?	Medium ?	id
Pest root test (18)	0.01	10	1×10^{-4}	0.01 to 10	Low	?	Poor	id
Sensitized oat and wheat coleoptile tests (this study)	5	0.5	2.5×10^{-3}	5 to 1,000	Medium	Low	Good	Requires little skill, little special equipment.
First internode test (this study)	1	0.5	5×10^{-4}	1 to 1,000	Medium to high	Low	Good	Requires care, rotating machine.

* For quantitative work.

** The accuracy with which an auxin concentration can be deduced from the measured growth response depends in part on: (a) the type of proportionality between auxin concentration and growth response (linear or logarithmic), (b) the slope of the curve representing this proportionality (a steep slope with large differences in growth corresponding to small changes in concentration tends to give more accurate results), (c) the limits of variability.

† In addition to a temperature-controlled darkroom which is required for best results in all the tests listed.

screening of possible test objects gave a rather discouraging answer as far as dicots are concerned (table II). On the other hand, monocots proved to be much more promising, for the first internodes of oat, sorghum and corn responded rather well to indole-3-acetic acid (IAA). The reason why sorghum and pop corn were tried is that they can develop first internodes up to 15 cm (sorghum) and 20 cm (corn) in length, whereas oat first internodes grow only to 6 cm (14). It was concluded that, among the test objects investigated, the coleoptiles and first internodes of grasses were the most promising ones, and work with other tissues was discontinued. Wheat and oat were selected as candidates for a coleoptile test, sorghum and oat for a first internode test.

On wheat seedlings, no first internode ever develops, even in total darkness. Such a species seemed interesting at first because 1) most of the varieties are hulless, and 2) no red light treatment is necessary to prevent the growth of the first internode. The batches of wheat seeds which were tried first (var. Ceres and Acadia) did not prove to be too sensitive and, also, gave a rather irregular growth with a relatively high standard error. Later, the wheat var. Genesee used by Powell (27) was tried and found to elongate very much at IAA concentrations superior or equal to 10 μgm/l, especially when the sections were soaked in water prior to the actual assay (see below, section 6). As far as oats are concerned, the problem of dehusking thousands of seeds had been solved previously by using hulless varieties, such as Brighton (25). The first internodes of this variety

TABLE II

SURVEY OF POSSIBLE TEST OBJECTS

PLANT	AGE IN DAYS	LOCATION OF SECTIONS	IAA CONC (μGM/L)	$G_{IAA} - G_0$**
Lepidium sativum †	3	Hypocotyl, 2 mm below curved part	10	0.08
		4 mm below curved part	10	0.23
		6 mm below curved part	10	0.27
Helianthus annuus	5	Hypocotyl (9.5-mm sections)	1	0.44
			10	0.99
			100	0.69
Phaseolus vulgaris var. Kentucky wonder	5	Hypocotyl (9.5-mm sections)	1	0.26
			30	1.32
			100	0.88
Z[illegible] var. Minhybrid white hullless, pop corn	4	1st internode	10	0.26
			50	0.72
			100	1.47
Triticum vulgare var. Ceres ††	3	Coleoptile, 3 mm below tip	1	0.27
			10	0.30
			100	1.02
Sorghum vulgare var. Western Blackhull kafir, F. C. 9098 ††	4	1st internode, 2 mm below node	1	0.05
			3	0.15
			10	0.53
			30	1.14
			100	1.66
			300	2.34
			1,000	1.95
Avena sativa	3	Coleoptile, 3 mm below tip †	10	−0.15
			100	0.55
			1,000	1.55
		1st internode, 3 mm below node	1	0.57
			3	0.74
			10	1.12
			30	2.24
			100	3.22
			300	3.51
			1,000	3.52

Ten 4-mm sections were grown in the dark for about 24 hours in 0.5 ml liquid (buffer * + 2 % sucrose) in each case, except for bean and sunflower seedlings in which instances ten 9.5-mm sections were grown in 1.0 ml.

* K_2HPO_4 (1.794 gm/l) + citric acid monohydrate (1.019 gm/l).

** This column gives the difference: (Growth in IAA) − (Growth without IAA).

† Exposed to 4 hours of red light during the first day of germination.

†† Obtained through the courtesy of the Agricultural Research Service, U.S.D.A.

proved to be more sensitive than those of sorghum and were finally selected as the best material.

MATERIALS AND METHODS

Oat seeds, var. Brighton, were soaked in tap water for two hours in the laboratory, then laid down on several layers of wet facial tissue (through which roots can grow freely) disposed at the bottom of glass or enamel trays. When coleoptiles were wanted, the trays with the seeds were then placed for 3 to 4 hours at about 35 cm below a concentric pair of G. E. white fluorescent "Circline" lights filtered through a Corning filter No. 2404. This illumination, devised by Thimann (unpublished) to produce an even light field of adequate intensity, suppresses the development of the first internode. The seeds were then grown in a darkroom maintained at 25° C with about 85 % relative humidity. When first internodes were wanted, the seeds were soaked in water for two hours in a closed container to protect them from light and grown in total darkness for 72 hours in moist clean maple sawdust. The subsequent manipulations were performed under the green light of a Westinghouse "green" fluorescent tube wrapped with three layers each of "amber" and "green" cellulose acetate window shading 0.0075 inch thick (obtained from the New England Plastic Shade Co., 80 Boylston Street, Boston, Mass.). This light source, devised by Dr. C. Yocum (unpublished), gives a light having mostly the wavelength of the 546 mμ mercury band which combines the following advantages: (a) it does not appreciably inhibit the growth of the first internode (12, 42); (b) it is phototropically inactive (17); (c) it is in the zone of the maximum sensitivity of the human eye.

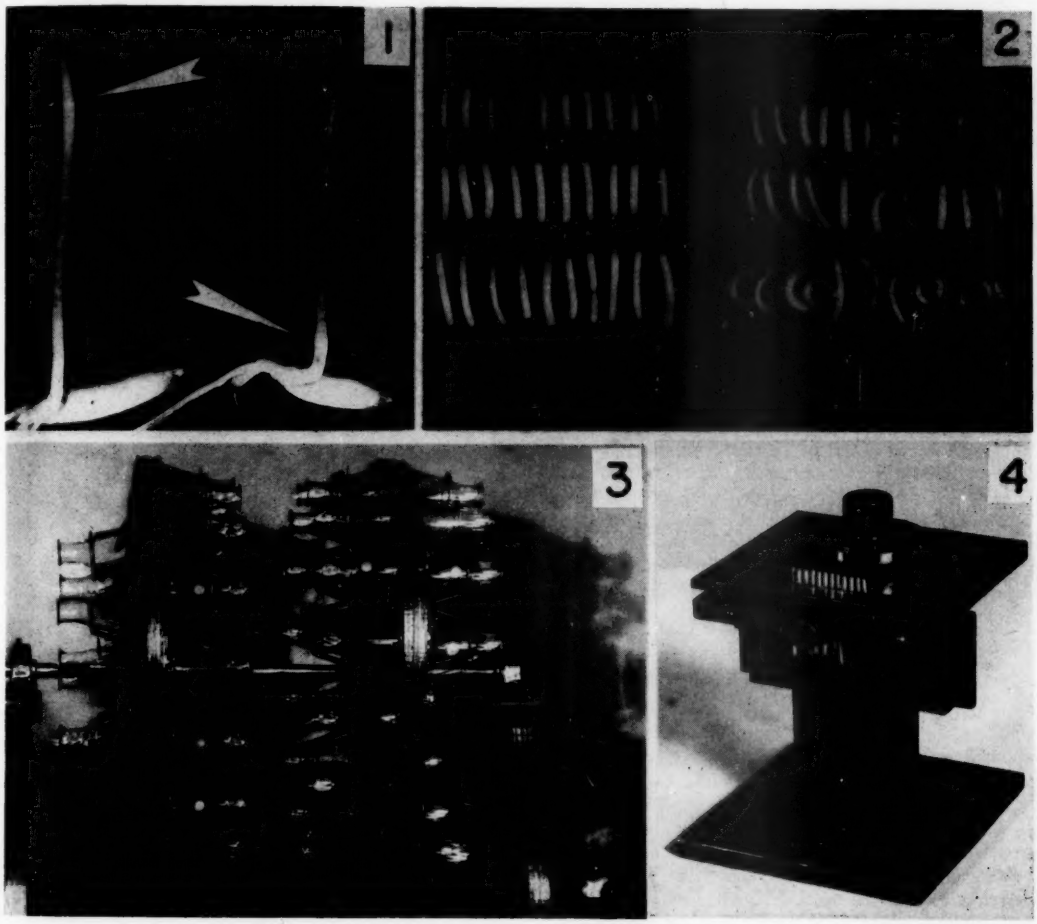
When the seedlings had reached the proper length (about 30 mm total length, fig 1), they were cut with Thimann's improvement of the coleoptile microtome (fig 4) of van der Weij (41). Thus, the error on the initial length of the sections was kept in the order of 0.04 mm.

In the case of coleoptiles, the enclosed first leaf was not removed to simplify the procedure and minimize the injury in handling the sections. Both Han-

cock and Barlow (15) and Bentley and Housley (6) consider that it is better to leave the primary leaf in the coleoptile section. Of course, this point does not apply to the first internodes which are made of solid stem tissue.

Ten replicates were used for each measurement. Generally, the coleoptile sections were floated 3 hours on large volumes (about 150 ml) of glass distilled water containing 1 mg/l of $MnSO_4 \cdot H_2O$ which increases the response to IAA (see below section 6) before being placed in the solutions to be tested. First internodes were presoaked in water for 1 hour only. During this time, they laid on the top of nets of cheesecloth stretched on plastic rings and kept breaking the surface of the liquid, a procedure similar to that devised by Hackett and Thimann (13).

A difficulty arose in the work with the first internodes because they curve very easily in auxin solutions. It was found that rotating the sections at about 1 rpm prevents curving and yields straight regular sections (fig 2). The rotating machine consists of vertical discs on which 125-ml Erlenmeyer flasks are fastened by means of springs (fig 3). The solutions and the sections are put in 13×100 mm Pyrex tubes which are placed in the flasks in which they remain at a slight angle above the horizontal plane. Coleoptile sections, on the contrary, did not need to be rotated, although we confirmed the report



FIGS. 1 to 4. FIG. 1. Oat seedlings, var. Brighton, at the right stages for use in the first internode test (*left*) and the coleoptile test (*right*). The arrows mark the position of the coleoptilar nodes. FIG. 2. Sections of oat first internodes of 4-mm initial length grown in sucrose 2 % + buffer without added IAA (*first rows*), with IAA (10 μgm/l) (*second rows*), and IAA (100 μgm/l) (*third rows*). The sections on the left were rotated, those on the right were not rotated: the latter ones curved. FIG. 3. The rotating machine used to rotate the oat first internodes. The Erlenmeyer flasks are ready to receive the tubes with the sections in 0.5 ml of solution. If the atmosphere is dry, the tubes are closed with vaccine stoppers. FIG. 4. Thimann's improvement of the van der Weij's coleoptile microtome, which allows exact work in the coleoptile and internode section tests.

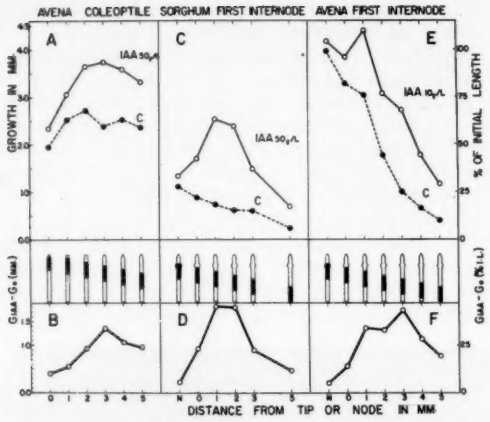


FIG. 5. The effect of location of the original 4-mm section on the growth in sucrose + buffer without added IAA (black circles, dotted lines) and with IAA (white circles, solid lines), and on the difference of these two elongations (curves B, D, and F). *Left*. Scales in mm. *Right*. Scales in % of the initial 4-mm length. On the abscissa of the curves C, D, E, and F, N means that the section includes the coleoptilar node. Each point is the mean of 10 replicates.

of Hancock and Barlow (15) that they grow more if rotated. In our experiments, the coleoptile sections floating on the solutions to be tested were gently shaken on a horizontal "Yankee rotator" shaker rotating at 23 rpm. In general, the sections, whether of coleoptiles or first internodes, were incubated in the test solutions for about 20 hours in the dark. After this time, they were measured to the nearest 0.1 mm by means of a binocular with an ocular micrometer.

Using these general conditions, a search was made to find out what factors would promote an optimal growth response to IAA.

Study of Some Factors Affecting the Response of the Bioassay to Indoleacetic Acid

Throughout the experiments which will now be reported, one must bear in mind that what we are looking for is the largest possible differential between the response to auxin and the response of the controls. In other words, a treatment which increases both the growth of the controls and that of the auxin-grown sections in the same amount does not increase the sensitivity of the test. Thus, in this investigation, the difference: *Growth in IAA – Growth without IAA* is of special importance. Also, we are aiming at a very sensitive bioassay. We will use, therefore, low concentrations of indole-3-acetic acid (10 μgm/l for first internodes, 50 μgm/l for coleoptiles).

1) Effect of the Location of the Section: One of the first points to determine with precision is where in the seedling one should cut the section to get the maximum response to IAA. To clarify this question, the following experiment was made. Four millimeter sections were cut at varying distances from either the tip of the coleoptile or the coleoptilar node and grown in sucrose and buffer (see the composition of the buffer used throughout these experiments at the end of section 9) with and without IAA. The results, represented in figure 5, show that the optimum response to IAA occurs when the 4 mm section is cut 3 mm below the tip of the oat coleoptile, or 1 to 2 mm below the coleoptilar node of sorghum seedlings, and 3 mm below the node of oat seedlings. Therefore, in all the subsequent experiments, the coleoptile sections were always cut 3 mm below the tip. In the case of first internodes, it was decided to cut the sections 2 mm below the node instead of 3 mm below because, in the latter case, the controls without IAA grow so little that they give no indication of inhibitors on paper chromatograms. One observes that first internode sections tend to grow considerably without added IAA if they are cut close to the node. In fact, they double in length if the node is included.

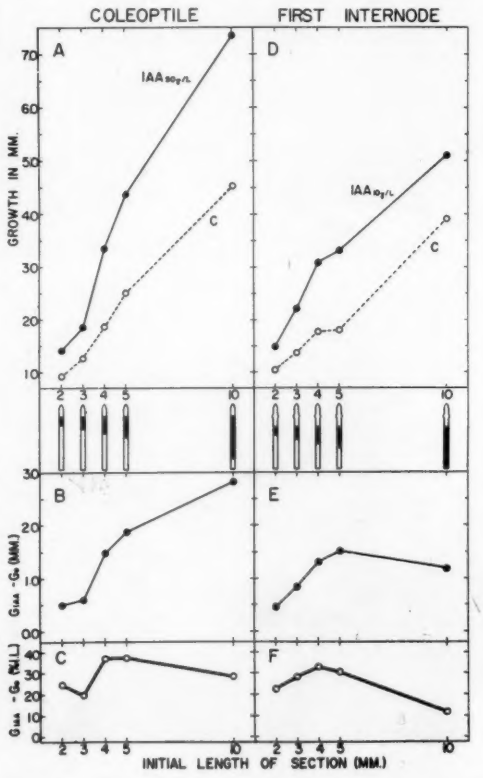


FIG. 6. Effect of the initial length of the oat sections on their growth in buffer + sucrose without added IAA (white circles, dotted lines) and with IAA (black circles, solid lines): graphs A and D. The other curves represent this effect on the difference $G_{IAA} - G_0$, expressed in mm (curves B and E) or in % of the initial lengths (curves C and F).

This effect is probably due to the fact that the meristematic zone at or just above the node functions as an auxin-producing center, a conclusion also arrived at by Mer (21). The fact that growth without added IAA decreases much more steeply in the case of internodes than in the case of coleoptiles as the section is cut further away from the node or tip, indicates that the actively growing zone is narrower in first internodes than in coleoptiles.

2) Effect of the Original Length of the Section: Starting at 3 mm below the coleoptile tip or at 2 mm below the coleoptilar node, we can now cut sections of 2, 3, 4, etc. mm of initial length and measure their growth in sucrose and buffer with and without IAA. The results, shown in figure 6, indicate that, generally speaking, the longer the initial section is, the larger is the growth. One can see also, however, that the longer the initial length of section, the larger is the growth of the controls without added IAA. The differential indicates an optimum of 5 mm of initial length in the case of oat internodes, beyond which no further gain can be obtained. This fact, again, indicates that the growing region of the internode is restricted to a narrow zone. In the case of coleoptiles, this is not the case because the difference $G_{IAA} - G_0$ continues to increase as the length of the initial section is extended. This justifies the use of 10-mm coleoptile sections recommended by many workers. However, the longer the sections, the less straight they are. In addition, if one considers the difference $G_{IAA} - G_0$ expressed in *% of the initial length,* then an initial length of 4 to 5 mm is better than a longer one, at least at the low auxin concentrations used here. For these reasons, an initial length of 4 mm was used throughout this work for both coleoptiles and internodes.

3) Effect of the Age of the Seedling: At what stage of development does the oat seedling give the maximum response to IAA? Many workers in the field have casually studied this point. It has been investigated here by taking into account not so much the actual length of time between soaking of seeds and sectioning, as the physiological stage of development apparent from the length of the coleoptile or internode. In general, the younger and shorter the

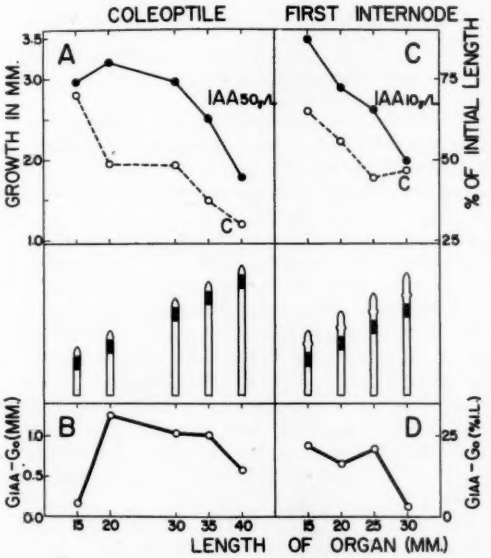
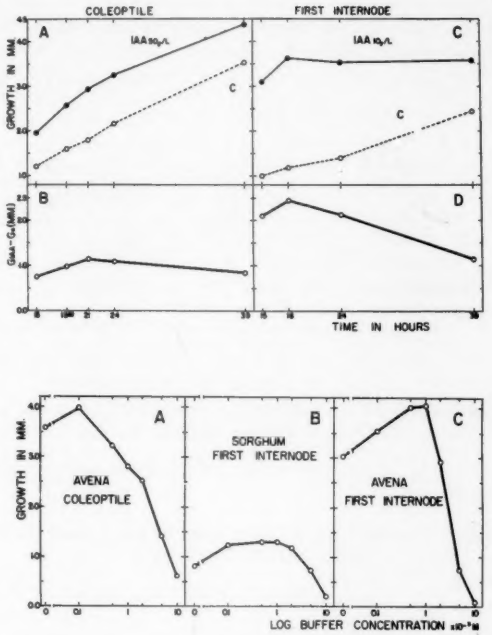


Fig. 7. Effect of the physiological age of the oat seedling (represented by the length of the coleoptile or of the first internode at the time of sectioning) on the growth of the sections in buffer + sucrose without added IAA (white circles, dotted lines) and with IAA (black circles, solid lines), and on the difference between these growths (curves B and D). Scales at the left are in mm, at the right in % of the initial 4-mm length.



Figs. 8 to 9. Fig. 8 (*above*). Effect of time in solutions on the growth of the sections in buffer + sucrose without added IAA (white circles, dotted lines) and with IAA (black circles, solid lines), and on the difference between these growths (curves B and D). Before time 0, the coleoptile sections had been presoaked for 3.5 hours in $MnSO_4 \cdot H_2O$ (1 mg/l) and the first internode sections for 1 hr in water. To exclude the effect of light during the measuring period, the experiment was done as follows: 5 replicate sets of 10 sections were started at the same time and one dish was measured and discarded at the various time intervals. Fig. 9 (*below*). Effect of the concentration of citrate-K phosphate buffer at pH 5.0 in the presence of 2% sucrose on the growth of 4-mm sections. The IAA concentration was 50 μgm/l in the case of Avena coleoptiles and 10 μgm/l in the cases of the first internodes of Sorghum and Avena.

TABLE III

EFFECT OF LIGHT ON THE IAA RESPONSE OF OAT COLEOPTILES AND INTERNODES

PLANT PART	TREATMENT	GROWTH OF CONTROLS	IAA CONC	GROWTH IN IAA	$G_{IAA} - G_0$
		mm	*μgm/l*	*mm*	*mm*
Oat coleoptiles	a) Cut under green * light, put for 5 hours in H_2O in the dark, then in the solutions	1.88	50	2.72	0.84
id	b) As a) but with a 30-min exposure to red light ** at the beginning of the 5 hrs in H_2O	1.76	50	2.41	0.65
id	c) As a) but with a 30-min exposure to red light ** while in the IAA solution	1.65	50	2.23	0.58
Oat coleoptiles	d) As a) but put for 5 hrs in buffer + sucrose instead of H_2O	1.78	50	3.43	1.65
id	e) As d) but with a 30-min exposure to red light ** at the beginning of the 5 hrs in buffer	1.42	50	3.06	1.64
id	f) As d) but with a 30-min exposure to red light ** while in the IAA solution	2.06	50	3.31	1.25
Oat coleoptiles	Cut under green * light, soaked for 3 hrs in $MnSO_4 \cdot H_2O$ in the dark and grown in the dark	2.00	10	2.21	0.21
			100	2.73	0.73
			1,000	4.16	2.16
id	Cut under red † light, the rest as above	1.69	10	1.80	0.11
			100	3.04	1.35
			1,000	4.57	2.88
Oat internodes	Cut under green * light, soaked for 1 hr in H_2O and grown in the dark	1.01	10	2.03	1.02
			100	3.26	2.25
			1,000	3.89	2.88
id	Cut under red † light, the rest as above	0.83	10	2.05	1.22
			100	2.85	2.02
			1,000	3.38	2.55

Ten 4-mm sections were used in all cases. Standard deviation from the mean: 0.15 to 0.25 mm for coleoptiles, 0.10 to 0.15 mm for first internodes.

* The green light was the same as that described under Material and Methods.

** This red light was that described under Material and Methods.

† This red light was different from the previous red light. It was obtained by filtering the light given by an incandescent bulb through a red filter transmitting radiations with wavelengths above 596 mμ.

organ is, the larger the growth (fig 7), but the growth of the controls is also high in the young stages. Thus the coleoptile sections taken from a 15-mm long coleoptile grow 75 % of their initial length without added IAA, almost as much as with 50 μgm/l of IAA. On the contrary, in the case of a 40-mm long coleoptile, the controls grow little, but so also do the sections treated with IAA. In short, the optimal differential response is obtained when either coleoptiles or internodes reach 20 to 25 mm in length. In the case of coleoptiles, seedlings up to 35 mm in length may still be used, whereas internodes of that length are too insensitive.

4) EFFECT OF TIME IN THE SOLUTIONS: After what time should one measure the elongation of the sections? This depends on the substance tested. In the case of low concentrations of IAA (fig 8) and in the presence of sucrose and buffer, the elongation of the coleoptile sections may continue for a long time. However, the elongation of the sections without IAA also increases in a parallel manner. The difference $G_{IAA} - G_0$ seems to be at its maximum about 20 hours after the sections have been put in the auxin solution. It is of interest to note that this difference does not increase with time as it does with larger volumes of solution (40 ml), higher IAA concentrations (1 mg/l), no buffer and no presoaking as used by Schneider (29). In the case of first internodes, growth seems to be completed after 18 hours (with low concentrations of IAA); if the sections are kept longer in the solutions, the controls grow more and more as time goes on, thus reducing the difference $G_{IAA} - G_0$. In consequence, if one wants to measure the IAA effect, at concentrations similar to those used here, one does not have to keep the sections in the solutions more than 20 hours. If one wants to measure inhibitors in relation to the controls without auxin, then the longer one waits, the larger will be the difference, because the controls will go on elongating for about two days. These long term experiments, however, may be interfered with by bacterial contamination. In the present investigation, a growth period of about 20 hours was used for both the coleoptiles and the first internodes.

5) EFFECT OF LIGHT: The experiments reported here were performed under the green light described above, with occasional exposure to very feeble intensities of red light due to other workers in the same darkroom. Since it has been reported (19, 30) that red light stimulates the growth of oat coleoptiles in the presence of IAA, an experiment was set up in which 30 min of red fluorescent light (the same light described in Materials and Methods) were given before and after the addition of IAA. As table III

shows, in no case was there a stimulation but rather a slight inhibition. On the other hand, a set of experiments in which the sectioning of the coleoptiles or internodes was done in duplicate under green fluorescent or red incandescent light gave different results (table III). Red incandescent light increased the growth of coleoptiles at concentrations of IAA of 100 μgm/l or above, but reduced it at lower concentrations. On the contrary, the same red light reduced the growth of first internodes in all cases. The discrepancy between the results obtained with the two different sources of red light indicates that the growth of coleoptiles may be affected in opposite ways according to the kind of red light they receive. To avoid this interference, only green light of the proper wavelength should be used.

6) Effect of Presoaking: We have tried to wash coleoptile and internode sections before placing them in the auxin solutions. It became immediately apparent that one hour washing in glass distilled water increases the response of the sections of both coleoptiles and internodes (table IV). It was reasoned that the system responsible for the inactivation of auxins at cut surfaces was in some way washed away. A series of possible inhibitors of this system and of reducing agents was tried (table IV). It was quickly found that sucrose, 2,3,5-triiodobenzoic acid or manganese would markedly increase the response of coleoptile tissues to IAA. The optimum concentration of Mn^{++} (as the sulfate) was about 5.9×10^{-6} M. The effects of Mn^{++} and sucrose given in pretreatment were not additive. The stimulatory effect of Mn^{++} ions on the growth of coleoptiles is well known (8, 10, 20, 32), but this seems to be the first instance demonstrating that a mere pretreatment with manganese increases the auxin response. The duration of the presoaking period is important. As figure 10 shows, the response of coleoptile sections to IAA decreases steadily as the presoaking time is extended over 3 hours. This is also true in buffer (graphs A and B, squares, fig 10). Sucrose, on the contrary, keeps the reactivity of the sections up and seems even to in-

TABLE IV

Effect of Various Pretreatments on the Response to IAA

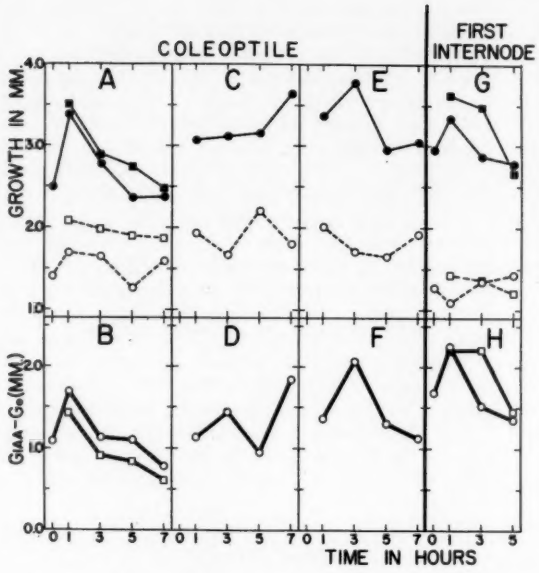
Plant part	Pretreatment	Time	Growth of controls	IAA conc	Growth in IAA	$G_{IAA} - G_0$
		hrs	*mm*	*μgm/l*	*mm*	*mm*
Oat first internodes	None	0	1.27	10	2.95	1.68
	H_2O	1	1.09	"	3.34	2.25
id	H_2O, floating	3	1.30	"	3.25	1.95
	" immersed	"	1.77 *	"	2.28	0.51
	2 % sucrose, floating	"	1.40	"	3.24	1.84
	" immersed	"	1.58	"	3.43	1.85
Oat coleoptiles	None	0	1.40	50	2.49	1.09
	H_2O	1	1.69	"	3.38	1.69
id	H_2O	3	2.00	"	2.47	0.47
	Ascorbic acid 10^{-5} M	"	1.74	"	2.78	1.04
	" 10^{-4} M	"	2.30	"	2.70	0.40
	Glutathione 10^{-4} M	"	2.22	"	2.65	0.43
	$Na_2S_2O_4$ 0.05 mg/l	"	1.99	"	2.39	0.40
	" 0.5 mg/l	"	2.26	"	2.47	0.21
id	H_2O	5	1.88	"	2.72	0.84
	$MnSO_4 \cdot H_2O$ 0.1 mg/l	"	3.00	"	3.98	0.98
	" 1 mg/l	"	2.54	"	4.64	2.10
	" 10 mg/l	"	1.93	"	3.46	1.53
id	$CoCl_2$ 0.01 mg/l	3	1.74	"	2.17	0.43
	" 0.1 mg/l	"	1.80	"	2.19	0.39
	" 1 mg/l	"	1.39	"	1.98	0.59
id	H_2O	3	1.22	"	1.90	0.68
	Sucrose 2 %	"	1.85	"	2.19 **	0.34 **
	$MnSO_4 \cdot H_2O$ 1 mg/l	"	1.59	"	2.57	0.98
	$MnSO_4 \cdot H_2O$ (1 mg/l) + sucrose 2 %	"	1.79	"	2.59	0.80
	2,3,5-Triiodobenzoic acid 0.05 mg/l	"	1.64	"	3.12	1.48
	" 0.5 mg/l	"	1.81	"	2.10	0.29
	Phenylacetic acid 1 mg/l	"	1.52	"	1.83	0.31
	" 10 mg/l	"	1.75	"	2.33	0.58
	" 100 mg/l	"	0.58 †	"	0.52 †	−0.06

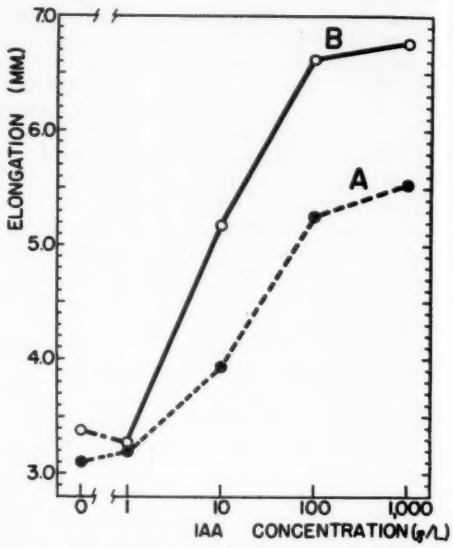
Ten 4-mm sections were used in each case. The sections were always breaking the surface, unless otherwise indicated. Standard deviation from the mean: 0.10 to 0.15 mm for first internodes, 0.15 to 0.25 mm for coleoptiles.

* Immersion in water increases the length of the controls somewhat, but the sections become more slender than when they are floated.

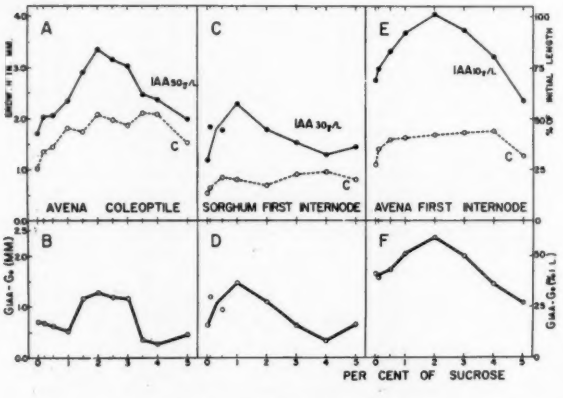
** In this particular series, the response to sucrose was abnormally low. It is generally of the order of the response to $MnSO_4$.

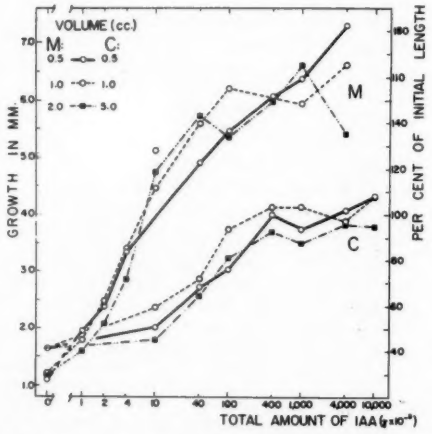
† The sections were dead at the end of the experiment.





FIGS. 10 to 11. FIG. 10 (*left*). Effect of various presoaking treatments on the growth of sections in buffer + sucrose without added IAA (dotted lines) and with IAA (solid lines). The IAA concentration was 50 μgm/l in the case of coleoptiles and 10 μgm/l for first internodes. The abscissa represent the time of presoaking before putting the sections in the final solutions with and without IAA. Curves A and B. Presoaking in water (circles) and in buffer (squares). Curves C and D. Presoaking in 2% sucrose. Curves E and F. Presoaking in $MnSO_4 \cdot H_2O$ (1 mg/l). Curves G and H. Presoaking in water (circles) and in buffer (squares). FIG. 11 (*right*). Effect of presoaking in glass-distilled water on the response to various IAA concentrations of 4-mm sections of wheat coleoptiles (var. Genesee). Black circles, dotted line—no presoaking. White circles, solid line—presoaking in water for 3 hours.



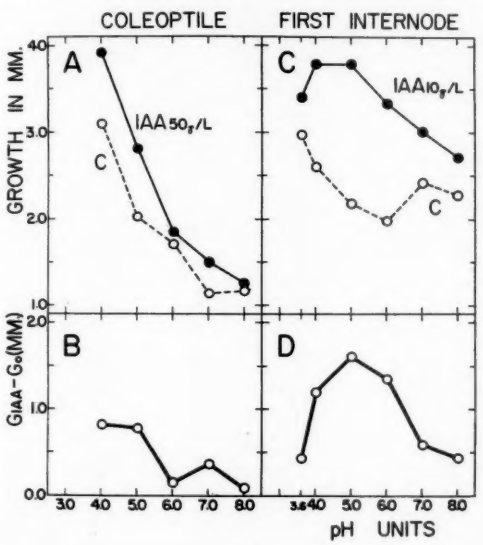


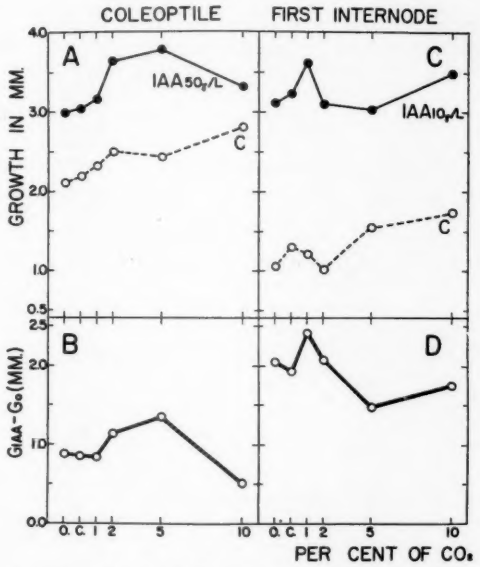
FIGS. 12 to 13. FIG. 12 (*left*). Effect of sucrose concentration on the growth of sections in buffer without added IAA (white circles, dotted lines) and with IAA (black circles, solid lines), and on the difference between these growths (curves B, D, and F). Scales in mm (*left*) and in % of the initial 4-mm length (*right*). FIG. 13 (*right*). Effect of the volume of liquid on the growth in various amounts of IAA of oat first internode sections (curves M) and coleoptile sections (curves C). The coleoptile sections were presoaked for 3 hrs in $MnSO_4 \cdot H_2O$ (1 mg/l) and the internode sections for 1 hr in H_2O. The internode sections were assayed in volumes of 0.5, 1.0 and 2.0 ml, the coleoptile sections in volumes of 0.5, 1.0 and 5.0 ml (buffer + sucrose + Tween 80, 0.1%). The growth of the controls of all three series is the same for first internodes and almost the same for coleoptiles.

crease it after 7 hours (graphs C and D), whereas the optimum presoaking time with manganese seems to be 3 hours (graphs E and F). In the case of first internodes, it is important that the sections be maintained so as to break the surface of the liquid; otherwise, their reactivity is decreased, except when sucrose is present (table IV). In the light of these findings, it was decided that the coleoptile sections would always be presoaked for 3 hours in 1 mg/l of $MnSO_4 \cdot H_2O$ and the internode sections for 1 hour in glass-distilled water. The response of wheat coleoptile sections to IAA is also markedly increased by a preliminary soaking period (see fig 11). In this case, floating the sections for 3 hours in water gave as good a result as floating them on solutions containing various manganese salts.

7) Effect of the Volume of Solution: If one desires a very sensitive test, one has to work not only with low concentrations but also with small volumes. The limiting factor, in the work with plant extracts, is the total amount of auxin available. Starting with given amounts of IAA, we have dissolved them in 0.5, 1.0, 2.0 ml (internodes) and 0.5, 1.0 and 5.0 ml (coleoptiles) of buffer + sucrose + "Tween 80" (0.1 %). The latter substance facilitates the dissolution of IAA in water and does not interfere with the growth of the sections; in fact, it favors it slightly. The coleoptiles were first presoaked 3 hours in manganese sulfate and the internodes 1 hour in water. The results, given in figure 13, demonstrate that there is hardly any benefit gained by using volumes larger than 0.5 ml. Large volumes have been reported to decrease the growth of the controls, perhaps because of the loss of some food factor (29). As figure 13 shows, this effect is completely eliminated when: 1) the sections are washed in large volumes of solutions before the actual test, and 2) sucrose is added to the test solutions. Accordingly, 0.5-ml volumes were used throughout this work and care was taken that no evaporation would take place during the assays. It should be mentioned also that, in the case of coleoptiles, we used flat bottom dishes instead of small beakers whose dome-shaped bottoms do not give a uniform depth of liquid with 0.5 ml.

8) Effect of Sucrose: Sugars, especially sucrose, have been shown by many authors to increase the response of oat coleoptile sections to IAA. On the contrary, Schneider (30) has claimed that sugar increases only slightly the growth of oat first internodes. We have re-examined the effect of sucrose on the elongation of both coleoptile and internode sections using always a series with and a series without IAA for each sucrose concentration. The results (fig 12) show that sucrose concentrations as low as 0.1 % stimulate growth of both the IAA-grown and the control sections. Moreover, it is clear that sucrose has a marked effect on the elongation of first internodes of sorghum and oat. This is at variance with Schneider's results, but it must be remembered that his testing conditions were different from ours (in particular, he used a dif-





Figs. 14 to 15. Fig. 14 (*left*). Effect of pH on the growth of sections in buffer + sucrose without IAA (white circles, dotted lines) and with IAA (black circles, solid lines), and on the difference between the two (curves B and D). Fig. 15 (*right*). Effect of the CO_2 concentration on the growth of 4-mm sections in buffer + sucrose without added IAA (white circles, dotted lines) and with IAA (black circles, solid lines), and on the difference between the two (curves B and D). On the abscissa, O. means open tubes, C. means closed tubes. Of course, the increasing concentrations of added CO_2 were done in closed tubes. Percent CO_2 actually means % of added CO_2. The CO_2 was added a few minutes after the sections had been introduced into the tubes.

ferent oat variety). The sucrose concentration which gives the largest differential response to IAA at low concentrations is 2 % for both oat coleoptiles and first internodes. In the case of sorghum internodes, it seems to be 1 % (fig 12). Wheat coleoptiles also respond well to sucrose; 0.5 % already causes a clear cut increase in length, but the optimum concentration is 2 %.

9) Effect of a Buffer: It is well known that the response of plant tissues depends on the number of undissociated auxin molecules present in the medium. Since an auxin such as IAA is a weak acid, it is important, in a quantitative test, to keep constant the concentration of undissociated molecules of such a substance. This can be done by regulating the pH of the solution. The effect of pH on the response of oat coleoptiles and first internodes to the same overall concentration of IAA is given in figure 14. Elongation decreases markedly as pH increases. Since the controls without added IAA also grow more at low pH's, the difference $G_{IAA} - G_0$ may not necessarily increase with decreasing pH. Figure 14 indicates that this difference is maximum at pH 5.0 for first internodes and close to this value for coleoptiles. A pH of 5.0 was, accordingly, chosen for all our experiments. Of course, the type of buffer used influences also the growth response. We employed here potassium phosphate-citrate buffers. They were chosen for the following reasons: 1) potassium stimulates auxin-induced growth (10, 37); 2) organic acids such as citric acid stimulate also the auxin-induced growth (36); 3) phosphate-citrate buffers have a good buffering capacity around pH 5.0. The effect of buffer concentration on the auxin-induced growth was also investigated (fig 9). In the case of oat coleoptiles, a concentration of IAA of 50μgm/l was used, whereas it was only 10 μgm/l in the case of sorghum and oat first internodes. A concentration of 10^{-3} M of the buffer slightly stimulates growth in coleoptiles, but higher concentrations rapidly inhibit it; in the case of internodes, the optimum lies around 10^{-2} M. Fearing that a 10^{-3} M concentration would not buffer adequately plant extracts separated on paper chromatograms, a concentration of about 10^{-2} M was used, the composition of this buffer being that previously reported (25), namely: K_2HPO_4 1.794 gm/l (about 10^{-2} M) + citric acid monohydrate 1.019 gm/l (about 5×10^{-3} M).

10) Effect of Inorganic Ions: Among the inorganic compounds which have been reported to increase auxin-induced growth are manganese (8, 10, 20, 32, 35), cobalt (22, 33, 35) and perhaps boron (16, 20). Table V shows that manganese sulfate definitely increases the growth of oat coleoptiles, but

Table V

Action of Inorganic Compounds

Plant part	Pretreatment	Solutions	Growth of controls	IAA conc	Growth in IAA	$G_{IAA} - G_0$
			mm	*μgm/l*	*mm*	*mm*
Oat coleoptile	H_2O, 5 hrs	Buffer + sucrose	1.94	100	2.91	0.97
	"	id + $MnSO_4 \cdot H_2O$ (1 mg/l)	2.43	100	4.30	1.87
	$MnSO_4 \cdot H_2O$, 5 hrs	Buffer + sucrose	2.27	100	4.02	1.75
Oat internode	H_2O, 1 hr	Buffer + sucrose	1.00	3	1.14	0.14
	"	id + $MnSO_4 \cdot H_2O$ (1 mg/l)	0.95	3	1.30	0.35
	"	Buffer + sucrose	1.06	10	3.11	2.05
	"	id + $MnSO_4 \cdot H_2O$ (1 mg/l)	1.10	10	2.74	1.64
	"	Buffer + sucrose	1.01	10	2.03	1.02
	"	id + $MnSO_4 \cdot H_2O$ 10 μgm/l	...	10	2.70	...
	"	100 μgm/l	...	10	2.55	...
	"	1,000 μgm/l	...	10	2.47	...
Oat internode	H_2O, 1 hr	Buffer + sucrose	2.23	10	2.89	0.66
	"	id + $CoCl_2$ 0.001 mg/l	1.14	10	2.27	1.13
	"	0.1 mg/l	1.23	10	2.65	1.42
	"	Buffer + sucrose	1.01	10	2.03	1.02
	"	id + $CoCl_2$ 0.01 mg/l	...	10	2.21	...
	"	0.1 mg/l	...	10	2.05	...
	"	1.0 mg/l	...	10	2.65	...
Oat coleoptile	$MnSO_4 \cdot H_2O$ (1 mg/l) 3 hrs	Buffer + sucrose	2.10	50	3.88	1.78
	"	id + boric acid 0.1 mg/l	2.43	50	2.38	−0.05
	"	1 mg/l	2.36	50	2.08	−0.28
	"	10 mg/l	1.91	50	2.18	0.27
Oat internode	H_2O, 1 hr	Buffer + sucrose	1.40	10	3.53	2.13
	"	id + boric acid 0.1 mg/l	2.02	10	3.20	1.18
	"	1 mg/l	1.60	10	3.05	1.45
	"	10 mg/l	2.08	10	3.02	0.94

Ten 4-mm sections were used in all cases. Standard deviation from the mean: 0.15 to 0.25 mm for coleoptiles, 0.10 to 0.15 mm for first internodes.

TABLE VI

EFFECT OF SOME ORGANIC ACIDS IN RESPONSE TO IAA

COMPOUND	CONC	COLEOPTILES				FIRST INTERNODE			
		GROWTH OF CONTROLS	IAA CONC	GROWTH IN IAA	$G_{IAA} - G_0$	GROWTH OF CONTROLS	IAA CONC	GROWTH IN IAA	$G_{IAA} - G_0$
		mm	*μgm/l*	*mm*	*mm*	*mm*	*μgm/l*	*mm*	*mm*
Buffer + sucrose		2.23	50	3.24	1.01	1.10	10	1.87	0.77
id + Na fumarate	5×10^{-5} M	2.32	"	3.05	0.73	0.82	"	1.99	1.17
id + "	5×10^{-4} M	2.44	"	3.15	0.71	1.52	"	1.99	0.47
id + Na malate	5×10^{-5} M	2.46	"	3.11	0.65	0.85	"	1.92	1.07
id + "	5×10^{-4} M	2.08	"	3.18	1.10	1.33	"	1.73	0.40
id + Na acetate	5×10^{-5} M	2.12	"	3.01	0.89	1.17	"	1.99	0.82
id + "	5×10^{-4} M	1.86	"	2.97	1.11	0.82	"	1.79	0.97

Ten 4-mm oat sections were used in all cases. The coleoptiles were presoaked for 3 hours in $MnSO_4 \cdot H_2O$ (1 mg/l), the first internodes for 1 hr in H_2O. They were then put in the test solutions which always contained the phosphate-citrate buffer described in the text (5×10^{-3} M of citric acid) and 2 % sucrose, with and without IAA. Standard deviation from the mean: 0.15 to 0.25 mm for coleoptiles, 0.10 to 0.15 mm for first internodes.

not always that of first internodes. Furthermore, it shows that a mere pretreatment with Mn^{++} ions before the addition of IAA also increases the growth of coleoptiles. The optimum concentration for such a pretreatment seems to be 1 mg/l of $MnSO_4 \cdot H_2O$ (see table IV), a concentration a hundred times less than that proposed by Bonner (8) in conjunction with arginine and high concentrations of IAA. Cobalt ions and boric acid gave variable results. Without added IAA, boric acid increased growth slightly, but decreased it somewhat in presence of this auxin.

11) EFFECT OF CO_2: Molliard (24) reported that 2 % CO_2 increases the elongation of lupin hypocotyls and pea stems in the light and in the dark. More recently, Yamaki (45) has found that the addition of IAA to oat coleoptile sections causes an immediate uptake of CO_2. We have also studied the action of various concentrations of CO_2 on the auxin-induced growth of the oat coleoptile and first internode. All the experiments were done in the presence of a buffer at pH 5.0, so that an acidification of the medium cannot be responsible for the results obtained. One series of experiments seemed to indicate that CO_2 increases somewhat the growth response to auxin, the optimum concentration being in the neighborhood of 5 % for coleoptiles and 1 % for first internodes (fig 15). However, the results which we have obtained, admittedly with the CO_2 added a few minutes after the IAA, were not always consistent.

12) EFFECT OF VARIOUS ORGANIC COMPOUNDS: We have examined a certain number of organic substances which may increase the auxin-induced growth. We can group some of them under the headings of organic acids, amino acids, vitamins.

An effect of organic acids was not expected since the buffer in which the sections are grown already

TABLE VII

EFFECT OF SOME AMINO ACIDS AND OF GLUTATHIONE ON THE RESPONSE TO IAA

COMPOUND	CONC	COLEOPTILES				FIRST INTERNODES			
		GROWTH OF CONTROLS	IAA CONC	GROWTH IN IAA	$G_{IAA} - G_0$	GROWTH OF CONTROLS	IAA CONC	GROWTH IN IAA	$G_{IAA} - G_0$
		mm	*μgm/l*	*mm*	*mm*	*mm*	*μgm/l*	*mm*	*mm*
Buffer + sucrose		2.03	50	2.81	0.78	0.92	10	1.89	0.97
id + L-arginine HCl	10^{-4} M	2.20	"	4.19	1.99	0.95	"	2.37	1.42
id + "	10^{-3} M	2.26	"	2.58	0.32	0.81	"	2.41	1.60
Buffer + sucrose		3.74	"	4.46	0.72	0.86	"	2.10	1.24
id + L-methionine	10^{-5} M	3.41	"	4.60	1.19	0.90	"	2.18	1.28
id + "	10^{-4} M	3.37	"	4.67	1.30	0.84	"	2.21	1.37
id + "	10^{-3} M	4.92	"	4.88	−0.04	0.76	"	2.37	1.61
Buffer + sucrose		2.23	"	3.24	1.01	1.10	"	1.87	0.77
id + glutamic acid	10^{-4} M	2.00	"	2.86	0.86	0.82	"	2.07	1.25
id + "	10^{-3} M	2.85	"	4.37	1.52	1.17	"	2.23	1.06
Buffer + sucrose		1.99	"	2.99	1.00	0.67	"	1.90	1.23
id + glutathione	10^{-4} M	2.28	"	2.65	0.37	0.75	"	1.73	0.98
id + "	10^{-3} M	2.56	"	3.94	1.38	0.74	"	2.32	1.58
id + "	3×10^{-3} M	2.89	"	5.03	2.14	0.89	"	2.24	1.35

Conditions as for table VI.

TABLE VIII

EFFECT OF VARIOUS VITAMINS ON THE RESPONSE TO IAA

COMPOUND	CONC	COLEOPTILES				FIRST INTERNODES			
		GROWTH OF CONTROLS	IAA CONC	GROWTH IN IAA	$G_{IAA} - G_0$	GROWTH OF CONTROLS	IAA CONC	GROWTH IN IAA	$G_{IAA} - G_0$
		mm	*μgm/l*	*mm*	*mm*	*mm*	*μgm/l*	*mm*	*mm*
Buffer + sucrose		2.23	50	3.24	1.01	1.10	10	1.87	0.77
id + thiamin HCl	10^{-5} M	2.15	"	3.12	0.97	0.97	"	1.54	0.57
id + "	10^{-4} M	2.28	"	3.32	1.04	0.96	"	1.99	1.03
id + riboflavin	10^{-6} M	2.18	"	3.51	1.33	1.48	"	1.72	0.24
id + "	10^{-5} M	1.99	"	3.13	1.14	1.01	"	1.80	0.79
id + Vitamin K_1	10^{-7} M	2.16	"	3.11	0.95	0.89	"	1.76	0.87
id + "	10^{-6} M	2.35	"	3.52	1.17	1.15	"	2.09	0.94
id + "	10^{-5} M	1.88	"	3.93	2.05	1.33	"	2.23	0.90
id + biotin	10^{-7} M	2.54	"	2.82	0.28	0.93	"	1.85	0.92
id + "	10^{-6} M	2.17	"	2.85	0.68	1.04	"	1.83	0.79
id + "	10^{-5} M	2.37	"	2.90	0.53	1.15	"	1.96	0.81
Buffer + sucrose		1.99	"	2.99	1.00	0.67	"	1.90	1.23
id + Ca pantothenate	10^{-6} M	2.31	"	2.82	0.51	0.78	"	1.76	0.98
id + "	10^{-5} M	1.99	"	2.87	0.88	0.65	"	1.91	1.26
id + folic acid	10^{-6} M	2.14	"	2.69	0.55	0.68	"	1.83	1.15
id + "	10^{-5} M	2.17	"	2.71	0.54	0.78	"	1.47	0.69
id + ascorbic acid	10^{-6} M	2.15	"	2.60	0.45	0.71	"	2.07	1.36
id + "	10^{-5} M	2.27	"	2.49	0.22	0.71	"	1.68	0.97
id + "	10^{-4} M	1.97	"	2.45	0.50	0.73	"	1.72	0.99
Buffer + sucrose		3.74	"	4.46	0.72	0.86	"	2.10	1.24
id + choline chloride	10^{-5} M	3.40	"	4.24	0.84	0.84	"	1.90	1.06
id + "	10^{-4} M	3.22	"	4.25	1.03	0.99	"	2.65	1.66
id + "	10^{-3} M	3.19	"	4.36	1.17	0.99	"	1.99	1.00

Conditions as for table VI.

contains about 5×10^{-3} M of citric acid. The results actually obtained did not show any real additional effect of fumarate, malate or acetate (table VI) which are the most stimulatory compounds of this group, as reported by Thimann and Bonner (36). Addition of calcium pantothenate in conjunction with acetate was without effect.

Among amino acids, arginine, methionine and glutamic acid, reported by Bonner (8) to increase the growth of the oat coleoptile, were very active on cole-

TABLE IX

EFFECT OF MISCELLANEOUS ORGANIC COMPOUNDS ON THE RESPONSE TO IAA

COMPOUND	CONC	COLEOPTILES				FIRST INTERNODES			
		GROWTH OF CONTROLS	IAA CONC	GROWTH IN IAA	$G_{IAA} - G_0$	GROWTH OF CONTROLS	IAA CONC	GROWTH IN IAA	$G_{IAA} - G_0$
		mm	*μgm/l*	*mm*	*mm*	*mm*	*μgm/l*	*mm*	*mm*
Buffer + sucrose		3.74	50	4.46	0.72	0.86	10	2.10	1.24
id + adenine sulfate	10^{-5} M	3.27	"	4.85	1.58	0.78	"	2.48	1.70
id + "	10^{-4} M	4.14	"	4.23	0.09	0.78	"	2.42	1.64
id + "	10^{-3} M	4.14	"	5.39	1.25	1.00	"	2.23	1.23
id + hypoxanthine	10^{-5} M	3.57	"	4.54	0.97	1.04	"	2.28	1.24
id + "	10^{-4} M	3.27	"	4.40	1.13	0.76	"	2.03	1.27
Buffer + sucrose		2.10	"	3.88	1.78	1.40	"	3.53	2.13
id + pectic acid	10 mg/l	2.23	"	2.90	0.77	1.60	"	3.15	1.54
id + "	100 mg/l	2.88	"	3.11	0.23	1.40	"	3.28	1.88
Buffer + sucrose		1.88	100	2.95	1.07	1.22	"	2.29	1.07
id + gibberellin X *	1 μgm/l	2.92	"	3.42	0.50	2.34	"	3.15	0.81
id + "	10 μgm/l	3.58	"	3.69	0.11	2.17	"	3.57	1.40
id + "	100 μgm/l	3.72	"	3.89	0.17	2.33	"	3.90	1.57
id + "	1,000 μgm/l	3.57	"	4.52	0.95	2.77	"	3.80	1.03

Conditions as for table VI.

* Kindly supplied by Dr. F. H. Stodola, U. S. Department of Agriculture, Peoria, Illinois.

optiles, somewhat less on first internodes (table VII). In fact, it seems that these amino acids by themselves, at high concentrations (10^{-3} M), stimulate the growth of coleoptiles without added IAA. This situation may create artifacts in the case of chromatograms of aqueous plant extracts, these amino acids acting like auxins. Fortunately, these substances alone are practically without effect on first internodes. Arginine, given only as a 3-hour pretreatment, did not have a marked effect on the growth of oat coleoptiles.

Glutathione at high concentrations (10^{-3} to 3×10^{-3} M) caused a large increase in the response to IAA of coleoptiles, somewhat less in first internodes. Part of this effect may be ascribed to its glutamic acid content since this latter substance is also active.

A series of vitamins were tried also. Some of the results obtained are listed in table VIII. Slight stimulations or inhibitions of growth were observed. For example, Vitamin K_1, stimulated growth somewhat and so did choline chloride in some experiments not reported here. On the whole, however, the vitamins tried here did not show any striking interference with the response to IAA.

Finally, among other miscellaneous organic compounds tested, adenine sulfate had some stimulatory effect, although not consistently. Hypoxanthine and pectic acid were without action, but crystalline gibberellin X had some auxin activity (table IX). A much longer list of compounds active on the growth of oat coleoptiles can be found in Avery and Sargent (3).

13) The Action of a Few Natural Auxins and Related Compounds: Having investigated a large number of variables affecting the response of the coleoptile and of the internode tests to IAA, we could then try to establish standard curves correlating growth response with auxin concentrations. In all cases, the coleoptile sections were presoaked in manganese and the first internode sections in H_2O. Then, the sections were incubated in various concentrations of the auxin to be tested with the phosphate citrate buffer described in section 9, 2 % sucrose and 0.1 % "Tween 80" to facilitate the dissolution of water-insoluble compounds such as the ethyl ester of IAA (IAE). If the results obtained with IAA are plotted on a semi-logarithmic graph (fig 16), it can be seen immediately that the response of the oat internodes to IAA is much more dramatic than that of coleoptiles. It can be seen further that the threshold of response is around $1 \times 5 \times 10^{-9}$ moles of IAA in 0.5 ml which is 0.875 μgm/l in that volume or about 4.3×10^{-4} μgm of actual IAA. This is about the threshold of sensitivity of the standard Avena curvature test. Finally, we can also see on figure 16 that the range of proportionality between elongation of internodes and IAA concentration is large (at least a hundred fold). The disadvantage is that the growth is proportional to the logarithm of the concentration, which diminishes the accuracy, as compared with the Avena curvature test in which the curvature is a linear function of the concentration.

How does the response to other auxins compare with that to IAA? Indoleacetonitrile (IAN), the ethyl ester (IAE) and the amide (IAam) of IAA were tried, as well as cis-cinnamic acid. Figures 17 to 20 give the results of these experiments. They show that, on a molar concentration basis, IAA, IAN, and IAE have similar activities, although, at certain concentrations, IAN and, especially IAE, are slightly more active than IAA. The results concerning IAN are surprising, considering the reports by Bentley and Housley (5) and Thimann (34) who found that IAN was at least 10 times more active than IAA on oat

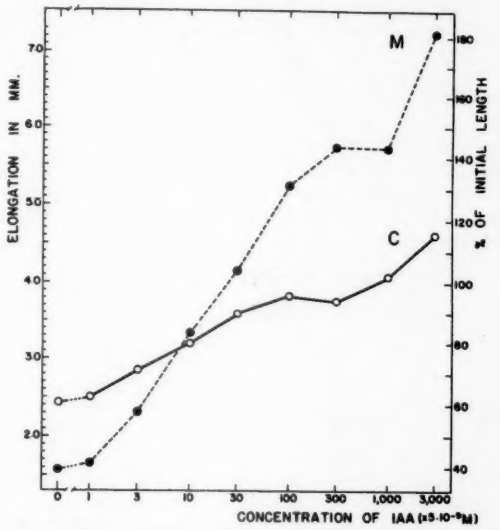
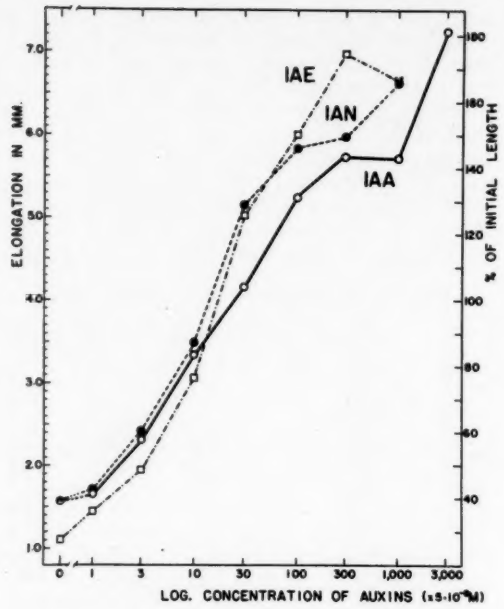
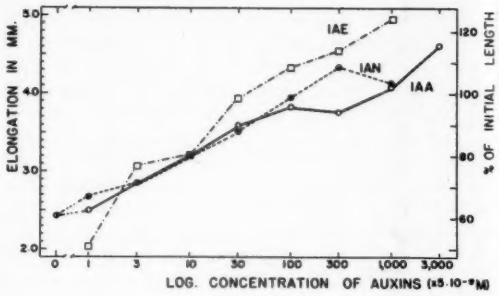
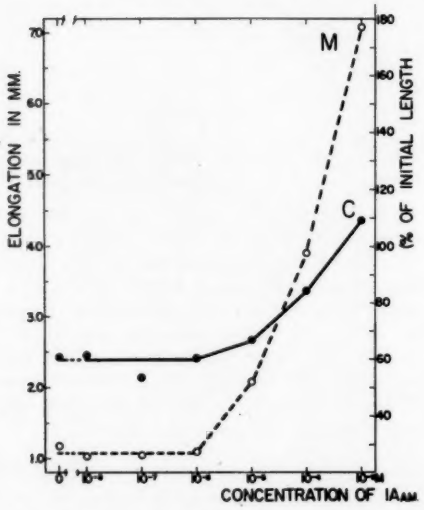


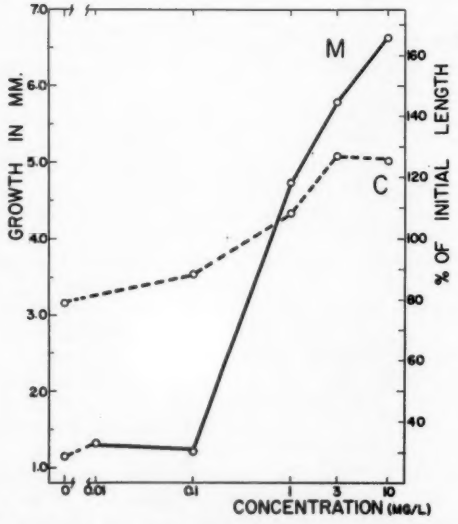
Fig. 16. Growth curves of oat coleoptile (C) and first internode (M) sections in buffer + sucrose + Tween 80 (0.1 %) with various concentrations of IAA. The scale at left gives the growth in mm over the initial 4 mm; the scale at right gives it in percent of the original length. The scale giving the IAA concentrations is logarithmic. The coleoptile sections had been floated 5 hrs on $MnSO_4 \cdot H_2O$ (1 mg/l) and the internode sections 1 hr on H_2O prior to being put into the auxin solutions in which they were left for about 20 hrs.

coleoptiles. This discrepancy may be due to a number of factors; difference in the variety of oat used, difference in the presoaking technique, the buffer, sucrose and Tween, etc. As far as IAam is concerned, it seems to show appreciable activity only at concentrations higher than 5×10^{-6} M in both coleoptiles and first internodes and seems to be from 50 to 100 times less active than IAA (fig 19). The lowest concentration of cis-cinnamic acid which gives an appreciable response seems to be 0.1 mg/l for coleoptiles (fig 20) which is about 6.7×10^{-7} M. Trans-cinnamic acid as well as its esters and hydroxy-derivatives such as ferulic and caffeic acids, were inactive on oat first internodes.









FIGS. 17 to 20. FIG. 17 (*top, left*). Growth curves of oat first internode sections in buffer + sucrose + Tween 80 (0.1 %) and various molar concentrations of indoleacetic acid (IAA), indoleacetonitrile (IAN) and the ethyl ester of IAA (IAE). Scales as in fig 16. The sections had been floated for 1 hr on H_2O before being put in the auxin solutions. FIG. 18 (*top, right*). Growth curves of oat coleoptiles (var. Brighton) in buffer + sucrose + Tween 80 (0.1 %) and varying molar concentrations of IAA, IAN, and IAE. The sections had been floated for 5 hrs on $MnSO_4 \cdot H_2O$ (1 mg/l) before being put in the auxin solutions. Scales as in fig 16. FIG. 19 (*bottom, left*). Growth curves of oat coleoptile (C) and first internode (M) sections in buffer + sucrose + Tween 80 (0.1 %) with various concentrations of indole-3-acetamide (IAam). The coleoptile sections had been pretreated for 3 hrs in $MnSO_4 \cdot H_2O$ (1 mg/l) and the internode sections for 1 hr in H_2O. FIG. 20 (*bottom, right*). Growth curves of oat coleoptile (C) and first internode (M) sections in buffer + sucrose + Tween 80 (0.1 %) with various concentrations of cis-cinnamic acid (1 mg/l = 6.7×10^{-6} M). Pretreatments as in fig 19.

Conclusions

Procedure for the Sensitized Coleoptile Cylinder Test: The steps can be summarized as follows:

1) Soak in tap water oat seeds, var. Brighton, or wheat seeds, var. Genesee, for 2 hours at room temperature.

2) Lay these seeds in parallel rows, the embryos up and pointing in the same direction, on several layers of wet facial tissue in glass or enamel trays.

3) Expose these trays for 3 to 4 hours to red light at 25° C. This step is omitted in the case of wheat seeds.

4) Grow the seeds at 25° C in a moist chamber in the dark for 3 days, the trays being tilted at about a 45° angle, the embryos pointing down. This procedure gives coleoptiles which are straight (G. Curry, private communication).

5) When the coleoptiles reach about 2.5 cm in length, select coleoptiles of equal lengths, cut 4 mm sections, 3 mm below the tip. Leave the primary leaf inside the sections.

6) Float these sections for 3 hours on glass-distilled water containing 1 mg/l of $MnSO_4 \cdot H_2O$. In the case of wheat, glass-distilled water alone is sufficient.

7) Put ten sections in 0.5 or 1 ml of the solutions to be tested, which should contain 2 % sucrose plus a buffer at pH 5.0 (K_2HPO_4 1.794 gm/l + citric acid monohydrate 1.019 gm/l).

8) Incubate about 20 hours in the dark at 25° C. A gentle horizontal shaking may yield a better and more uniform growth. Rotating the tubes around a horizontal axis at about 1 rpm improves growth even more.

9) Measure the length of the sections under a binocular with ocular micrometer, or by projecting their shadows through a photographic enlarger, or by any other suitable means.

Procedure for the First Internode Test: 1) Soak in tap water oat seeds, var. Brighton, for 2 hours at room temperature in a closed, light-tight container.

2) Lay the seeds on about 1 inch of moist, clean sawdust (maple sawdust is good), and cover with about ½ inch of moist sawdust. These operations should be performed under dim light, preferably green light (546 mμ).

3) Grow the seeds at 25° C for 3 days (in sawdust) in complete darkness. From then on, all manipulations should be performed under green light.

4) When the first internodes reach about 2.5 cm in length, they are ready to be used. The coleoptiles, at that time, should be only 0.5 cm long. Longer coleoptiles indicate that the growing seedlings have received light of active wavelengths. Cut 4-mm sections, 2 mm below the coleoptilar node.

5) Wash the sections in glass-distilled water for 1 hour during which the sections should break the surface of the liquid, for example by laying on cheesecloth stretched on plastic rings.

6) Put ten sections per one 13 × 100 or 16 × 125 mm Pyrex tube with 0.5 or 1 ml of the solution to be tested containing the same buffer (1.794 gm/l of K_2HPO_4 + 1.019 gm/l of citric acid monohydrate) and the same sucrose concentration (2 %) as for coleoptiles.

7) Place each tube in an Erlenmeyer flask and rotate around a horizontal axis at about 1 rpm in the dark for about 20 hours, at 25° C.

8) Measure the length of the sections to 0.1 mm.

Value of the Tests Proposed: At the beginning of this article, we have enumerated the qualities that a good bioassay for auxins should have. Let us see if the two tests proposed meet these criteria. A major drawback of the ordinary straight growth coleoptile test was its relatively low sensitivity. By carefully studying the variables that affect this test, we are now able to detect 5 μgm/l of IAA in 0.5 ml instead of 10 μgm/l in 10 ml, which represents a forty fold increase in sensitivity. However, by using the first internode, we can reach a 200 fold increase in sensitivity over the cylinder test as described by Bentley (4). The proportionality range of these tests is good, although the semi-logarithmic relationship between growth and auxin concentration reduces the accuracy. The specificity for auxins is reasonable, but certain substances such as arginine, glutamic acid, methionine, glutathione, etc. also stimulate to some extent the growth of coleoptile sections. First internodes, on the whole, appear to be more specific in this respect. The reliability is good. It should be mentioned that the first internode test gives results that are more uniform statistically than the coleoptile test which has a higher standard error. The standard deviation from the mean is usually 0.10 to 0.15 for first internodes, 0.15 to 0.25 for coleoptiles. Finally, the tests are not too difficult to perform, although the results reflect the care taken in selecting uniform seedlings, cutting the sections exactly where they should be cut and of exactly the same length. The first internode test requires the rotating of the sections, however, but not a humidity-controlled room as does the Avena curvature test. The use of internodes does away with the problem of the primary leaf in coleoptiles. The first internodes have been reported to grow both by cell division and cell enlargement (2) but Schneider (30) concluded that the elongation of sections under the influence of IAA was due to cell enlargement almost exclusively. The first internode test has been used with success in the assay of numerous auxin chromatograms. In particular, it has enabled us to detect clearly the natural auxins present in an extract of as low as 300 mg of fresh plant material (26). Finally, the first internode test is much less sensitive to inhibitors than the coleoptile test, which enables it to clearly detect auxins in an impure extract in which substances inhibitory to coleoptiles would seriously interfere with the auxin picture.

Summary

After a preliminary screening for a good test object for the assay of auxins, the coleoptile of wheat and oat and the first internode of oat have been re-

tained. Among the factors affecting the magnitude of the response of these test objects to indole-3-acetic acid, the following have been studied: the location of the section on the seedling, the length of the initial section, the age of the seedling, the length of time in the auxin solution, the quality of light, the pretreatment in various solutions, the volume of the solution, the concentration of sucrose, the pH and concentration of a citrate-phosphate buffer, inorganic ions, CO_2 concentration, certain organic acids, amino acids, vitamins, auxins, etc. These investigations resulted in the working out of two tests: (a) a more sensitive oat or wheat coleoptile cylinder test, and (b) a new straight growth test using the oat first internode, which is as sensitive as the Avena curvature test of Went.

The authors are indebted to both Professor R. H. Wetmore and Professor K. V. Thimann for the help, facilities and encouragement which they generously provided. They wish to thank also the Canadian Department of Agriculture, Central Experimental Farm, Ottawa, Canada, for its complete cooperation and the gift of oat seeds. The hulless oat var. Brighton can be obtained from the Canadian Department of Agriculture, Experiment Station, Scott, Saskatchewan, Canada. The pure indole-3-acetonitrile used here was synthesized in Professor E. R. H. Jones' laboratory and obtained through the courtesy of Professor K. V. Thimann. The sample of indole-3-acetamide was kindly supplied by Professor R. L. Wain's laboratory, that of cis-cinnamic acid by Dr. J. Van Overbeek and that of gibberellin by Dr. F. H. Stodola.

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PATHWAYS OF OXIDATION IN CELL-FREE POTATO FRACTIONS[1,2]

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Knowledge of the fate and mode of transport of the electrons involved in energy-yielding oxidations is essential for an understanding of physiological activity. Recently, we have studied this problem with special reference to terminal oxidases and plant growth (7, 8). Particular attention was paid to respiratory mechanisms in the potato tuber (31). In the present study, oxidative pathways in the potato were examined by determining the activities of enzymes in isolated cell fractions.

It is now generally assumed that respiration, both in higher plants and animals, is mediated by a series of electron carrier systems, including the pyridine nucleotides, flavoproteins, and cytochromes. Examination of the oxidation-reduction potentials of these systems indicates that the major portion of the energy that is available in organic substrates is released between the level of reduced pyridine nucleotide and molecular oxygen. In view of the importance of these reactions, an attempt was made to reconstruct the pathway of electron transfer from pyridine nucleotide to oxygen; succinate oxidation has also been examined. Studies of this type on isolated animal mitochondria (25) have revealed a characteristic electron pathway from pyridine nucleotide to flavin, thence via an unknown factor to the cytochrome system, and thence to oxygen. Many of these components have been demonstrated in plants, but evidence for the whole sequence of reactions in a single preparation has not been reported. Since plant mitochondria are centers of cellular respiration (16), this study deals primarily with activities associated with isolated particles; some experiments with the soluble fraction are also included. Enzyme activities were determined by following changes in absorption with a spectrophotometer.

MATERIALS AND METHODS

Potato tubers (*Solanum tuberosum*, var. Katahdin) were received monthly from Maine and stored at 10° C. The preparation of cell fractions was carried out in a 4° C cold room. Ten grams of peeled tissue were ground in a glass mortar with sand and an equal volume of 0.05 M phosphate buffer (pH 7.0) which was also 0.2 M with respect to sucrose. After filtering through cheesecloth, the homogenate was centrifuged at 2,000 × g for 10 minutes, the sediment discarded, and the supernatant centrifuged at 10,000 × g for 30 minutes. The resulting supernatant fraction (SF) was poured off; the sediment was washed by resuspending in 15 ml sucrose-phosphate buffer and recentrifuging at 10,000 × g for 30 minutes. The washed sediment was resuspended in 1 to 3 ml of sucrose-phosphate solution. This suspension of particulate material is referred to as the mitochondrial preparation (M_w), on the basis of the centrifugal

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force used to sediment the particles and the enzyme complement of this fraction.

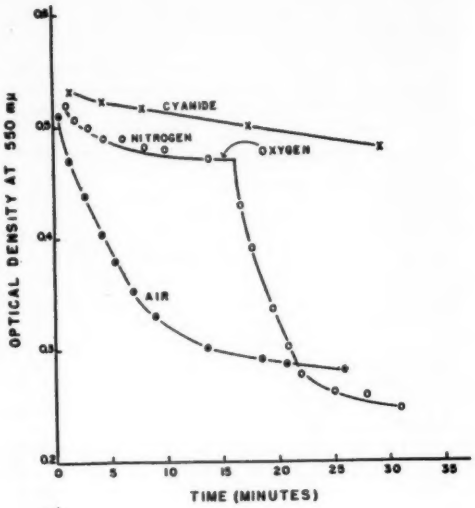
Reduced diphosphopyridine nucleotide (DPNH) and cytochrome c were purchased from the Sigma Chemical Company. Reduced cytochrome c was prepared by reduction with sodium hydrosulfite followed by aeration to remove the excess. Antimycin-A (AA) was obtained through the courtesy of Dr. G. W. Keitt; solutions of this compound were made up in 95 % ethyl alcohol. All gases were purchased commercially.

Reactions were performed at room temperature in cuvettes of one cm light path, and a Beckman Model DU spectrophotometer was used to follow changes in optical density. Cytochrome c was measured at 550 $m\mu$ and DPNH at 340 $m\mu$. Enzyme solution was added to the cuvette with a hypodermic syringe. When reactions were carried out in a gas phase other than air a vaccine stopper was fixed on to the cuvette, which was then flushed with gas. Solutions used in these reactions were equilibrated with the appropriate gas before transferring (with syringe) to the flushed cuvette. Where enzyme rates were calculated, the earliest linear portions of the time course curves were used.

Results

Cytochrome Oxidase: In both plant and animal cells this enzyme is associated with the mitochondria (6), and its localization on potato particles has already been established (5). That the mitochondrial preparation used in this study possesses an active cytochrome oxidase is shown in figure 1. The oxidation of reduced cytochrome c does not proceed in an anaerobic atmosphere but goes rapidly when oxygen is added. As would be expected, the enzyme is very sensitive to cyanide; in four experiments, the oxidase was inhibited an average of 95 % by 1×10^{-4} M cyanide (fig 1). The effects of two other inhibitors, carbon monoxide (CO) and Antimycin-A, on the ability of the particles to oxidize cytochrome c are indicated in figure 2. In the dark, a 19/1 CO/O_2 mixture inhibited the oxidase 70 % relative to the appropriate N_2/O_2 control; 1 μg/ml AA caused no inhibition. These results agree with the classical observations of CO as a cytochrome oxidase inhibitor, and with the finding that the cytochrome oxidase of animal tissues is insensitive to AA (18). We are not dealing with a cytochrome c peroxidase, since this enzyme is insensitive to CO (27).

DPNH Oxidase: When a suspension of particles is added to a solution containing DPNH, the optical density at 340 $m\mu$ decreases. Since this reaction does not go in nitrogen and depends on a supply of oxygen (fig 3), the system exhibiting this activity is referred to as DPNH oxidase. Several investigators have noted a marked dependence of the DPNH oxidase system of isolated animal mitochondria on such experimental conditions as phosphate concentration (25) and tonicity (12). With potato particles, a brief suspension in distilled water did not significantly increase the oxidase activity. Omission of sucrose from the



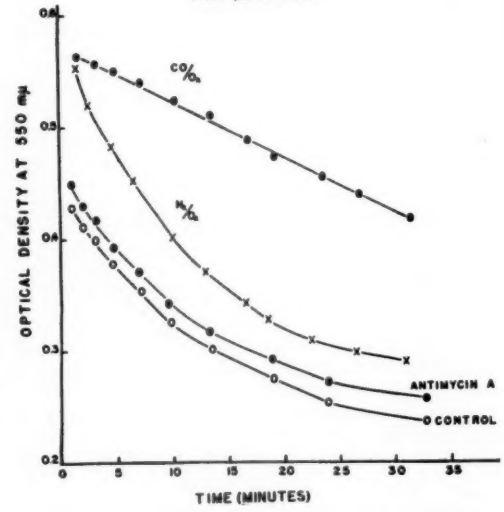


Fig. 1 (*above*). The oxidation of reduced cytochrome c by a mitochondrial preparation. All cuvettes contained 2×10^{-5} M reduced cytochrome c, 0.2 M sucrose, 0.01 M phosphate buffer (pH 7.0), and 0.1 ml M_w in a total volume of 3.0 ml. Solid circle—Control, in air. Crosses—Cyanide, with 1×10^{-4} M NaCN. Open circle—Nitrogen as gas phase, then flushed with O_2.

Fig. 2 (*below*). The effect of carbon monoxide and Antimycin-A on the mitochondrial cytochrome oxidase. All cuvettes contained cytochrome c, sucrose, buffer, and M_w as in figure 1. Open circle—Control, with 0.03 ml 95 % alcohol. Solid circle—Antimycin-A, with 0.03 ml 95 % alcohol plus 1 μgm/ml AA. Crosses—19/1 N_2/O_2 as gas phase. Crossed circles—19/1 CO/O_2 as gas phase, in the dark.

reaction mixture or increasing the phosphate concentration to 0.05 M promoted the activity roughly 50 %. Such effects are probably related to the morphological integrity or permeability of the particles.

Inhibitors were used to identify the components of this system. The DPNH oxidase can be almost completely blocked by 1×10^{-3} M cyanide (fig 4). In four experiments, this concentration of cyanide caused an average inhibition of 94 %. This suggests strongly the participation of a heavy metal-containing terminal oxidase. As shown in figure 4, Antimycin-A is also an effective inhibitor; in three experiments 1 μg/ml AA caused 88 % inhibition. (In separate runs it was established that the amount of alcohol included in the control had no significant effect.) We can conclude that most of the DPNH oxidation by the particles is mediated by an AA-sensitive factor and a cyanide-sensitive reaction.

The oxidation of DPNH by the mitochondrial preparation is significantly promoted by cytochrome c. As indicated in table I, 2×10^{-5} M cytochrome c more than doubles the rate, and higher concentrations

TABLE I

EFFECT OF CYTOCHROME C ON DPNH OXIDATION

CYTOCHROME CONC	INITIAL RATE
M	$\Delta OD/min$
0	5.2×10^{-3}
2×10^{-5}	11.0×10^{-3}
5×10^{-5}	9.0×10^{-3}
1×10^{-4}	10.8×10^{-3}

Reaction mixture contained 5×10^{-5} M DPNH, 0.2 M sucrose, 0.01 M phosphate buffer (pH 7.0), 0.25 ml M_w (equivalent to 0.83 gm fresh wt), and was made up to 3.0 ml with water and cytochrome c solution. Decrease in optical density measured at 340 mμ.

do not give any additional stimulation. In the presence of added cytochrome, the DPNH oxidation is much less sensitive to Antimycin-A; in one experiment, 1 μg/ml AA inhibited the oxidase system only 40 %. This suggests that the exogenous cytochrome c provides an alternative pathway of oxidation which can by-pass the AA-sensitive factor.

DIAPHORASE: Both cyanide and Antimycin can almost completely block the oxidation of DPNH by the mitochondria. In the presence of either of these inhibitors, the addition of a redox dye, such as methylene blue, is followed by the rapid disappearance of the 340 mμ absorption band, indicating the oxidation of the DPNH. In figure 5 the effect of 2,6-dichlorophenol-indophenol (DIP) on the AA-blocked system is shown. The rate of DPNH oxidation is at least four times greater with DIP acting as the hydrogen acceptor (ΔOD/min. of 0.03) than with molecular oxygen (ΔOD/min. of 0.007). This dye is more effective in the reaction than is methylene blue. The results indicate that there is a very active diaphorase on potato mitochondria. The enzyme acts above the level of those steps in the DPNH oxidase system which are sensitive to Antimycin and cyanide. With both dyes there is a slow non-enzymatic oxidation of DPNH in the absence of the particles (fig 5).

DPNH-CYTOCHROME C REDUCTASE: The reduction of cytochrome c by potato mitochondria can be followed if the cytochrome oxidase activity of the particles is blocked with an inhibitor such as cyanide. In the absence of added substrate oxidized cytochrome c is not reduced. However, if DPNH is present in the reaction mixture the addition of mitochondria is followed by a rapid increase in the optical density at 550 mμ (fig 6). Since this cytochrome reduction is accompanied by a simultaneous decrease in absorption at 340 mμ it is clear that DPNH-cytochrome c reductase activity is being measured. There is no reaction in the absence of the mitochondria. As shown in figure 6, the reductase activity is only moderately diminished by 1 μg/ml Antimycin-A, which in two experiments inhibited 21 and 33 %. This supports the conclusion that the major pathway between DPNH and added cytochrome c does not involve the AA-sensitive factor.

SUCCINIC-CYTOCHROME C REDUCTASE: The ability of potato mitochondria to utilize succinate as a substrate for the reduction of cytochrome c is also shown in figure 6. The succinic–cytochrome c reductase activity is roughly half of that observed with DPNH as substrate. In addition, there is an initial "lag period" before the maximum rate is reached. No such delay was observed in the DPNH–cytochrome c reductase reaction, which is maximal within half a minute after the addition of particles. These observations may be related to differences in the accessibility of the two substrates to the oxidizing systems. A further difference is indicated by the great sensitivity of succinic–cytochrome c reductase to Antimycin; figure 6 shows a 93 % inhibition by 1 μg/ml AA. The AA-sensitive factor must be located directly on the oxidative pathway between succinate and cytochrome c.

RELATIVE RATES: In the above characterization of the enzymes associated with potato particles, absolute rates of reaction have not been considered. To compare the several activities, all of the enzymes were assayed with the same mitochondrial preparation, and the results are shown in table II. Rates are calculated in terms of the initial fresh weight of tissue.

TABLE II

COMPARISON OF RATES OF OXIDATION AND REDUCTION OF CYTOCHROME C AND DPNH BY MITOCHONDRIAL ENZYMES

ENZYME	ASSAYED	MICROMOLES $\times 10^{-3}$/MIN $\times$ GM FW OF WHOLE TISSUE
Cytochrome c oxidase	Cyt c	13.88
DPNH oxidase	DPNH	1.95
Diaphorase	DPNH	9.75
DPNH–cytochrome c reductase	Cyt c	3.57
Succinic–cytochrome c reductase	Cyt c	1.86

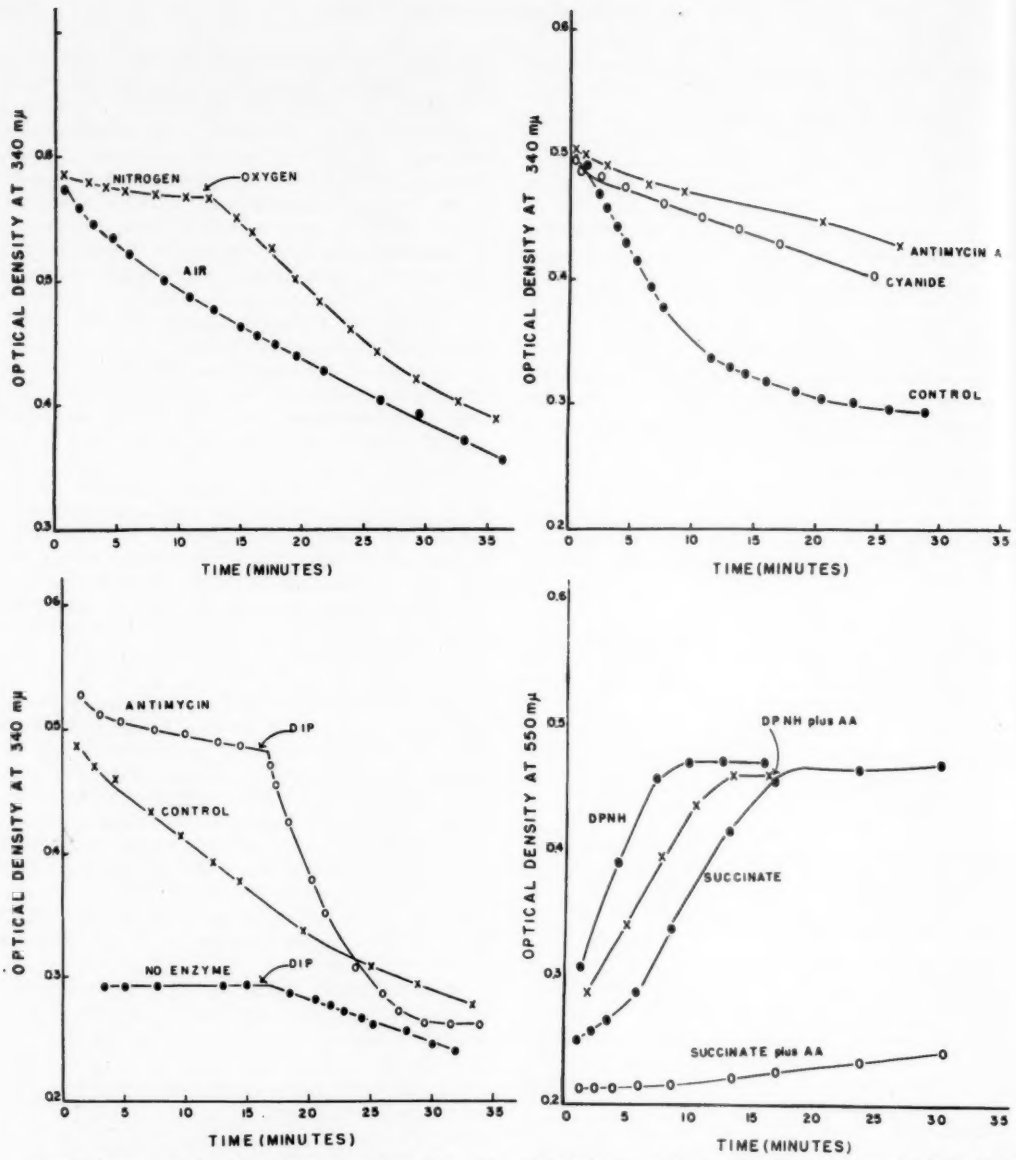


FIG. 3 (*upper, left*). The DPNH oxidase of a mitochondrial preparation. All cuvettes contained 5×10^{-5} M DPNH, 0.2 M sucrose, 0.01 M phosphate buffer (pH 7.0) and 0.25 ml M_w in a total volume of 3.0 ml. Solid circle—Control, in air. Crosses—Nitrogen as initial gas phase, then flushed with oxygen.

FIG. 4 (*upper, right*). The effect of cyanide and Antimycin-A on the mitochondrial DPNH oxidase activity. All cuvettes contained DPNH, sucrose, buffer, and M_w as in figure 3. Solid circle—Control. Open circle—Cyanide, with 1×10^{-3} M NaCN. Crosses—Antimycin-A, with 1 μgm/ml AA.

FIG. 5 (*lower, left*). The diaphorase activity of a mitochondrial preparation. All cuvettes contained 5×10^{-5} M DPNH, 0.2 M sucrose, and 0.05 M phosphate buffer (pH 7.0) in a total volume of 3.0 ml. Crosses—Control, with 0.25 ml M_w and 0.03 ml 95% alcohol. Open circle—Antimycin, with 0.25 ml M_w and 1 μgm/ml AA. Solid circle—No enzyme, with 1 μgm/ml AA. At the arrow, 0.03 ml of 10^{-3} M 2,6-dichlorophenol-indophenol (DIP) was added.

FIG. 6 (*lower, right*). DPNH-cytochrome c reductase and succinic-cytochrome c reductase activity of potato mitochondria. All cuvettes contained 2×10^{-5} M cytochrome c, 0.2 M sucrose, 0.01 M phosphate buffer (pH 7.0), 0.001 M NaCN, and 0.25 ml M_w in a total volume of 3.0 ml. Crossed circle—Contained 5×10^{-5} M DPNH and 0.03 ml 95% alcohol. Crosses—5×10^{-5} M DPNH and 1 μgm/ml AA. Solid circle—0.015 M succinate and 0.03 ml 95% alcohol. Open circle—0.015 M succinate and 1 μgm/ml AA.

In the course of a large number of experiments the individual reaction rates varied considerably, probably due in part to differences in the number of mitochondria recovered. The data of table II indicate in a general way the relative activities of the enzymes. In comparing rates it must be remembered that the oxidation of one mole of DPNH corresponds to two moles of cytochrome, since oxidation or reduction of the latter involves only one electron. Considering the high activity of diaphorase and cytochrome oxidase, it seems likely that the oxidation of DPNH by these particles is limited by the rate of reduction of cytochrome c.

Enzymatic Activities of the Supernatant: In order to obtain some indication as to the intracellular distribution of the above enzymes, experiments were carried out with the supernatant fraction (SF) remaining after centrifugation at $10{,}000 \times g$. The fact that the SF is essentially unable to oxidize reduced cytochrome c confirms the mitochondrial localization of cytochrome oxidase. On the other hand, addition of a small volume of SF to a DPNH solution results in a rapid decrease in the absorption at 340 mμ; rates between 300 and 700×10^{-4} micromoles DPNH/min × gm fresh weight (FW) were recorded. The nature of the system in the SF which brings about this extremely rapid attack on DPNH has not been worked out, although some preliminary experiments have been done. The reaction is presumably enzymatic, since boiling the supernatant destroys this activity. Oxygen is apparently not involved, since the reaction proceeds at an equal rate under anaerobic conditions; nevertheless, 1×10^{-3} M cyanide caused roughly a 50 % inhibition in two experiments.

The addition of supernatant to a solution of oxidized cytochrome c is followed by an increase in absorption at 550 mμ (fig 7). The ability of the SF alone to reduce cytochrome c varied widely in several experiments. Figure 7 shows that the reaction is not affected by the presence of succinate, indicating that essentially all of the succinic–cytochrome c reductase system is on the mitochondria; this is also the case in Pisum homogenates (28). However, addition of DPNH to the reaction mixture greatly accelerates the cytochrome reduction (fig 7). The activity of the DPNH–cytochrome c reductase system (more than 200×10^{-4} micromoles cytochrome c/min × gm FW) in the supernatant is roughly ten times greater than that of the particulate enzyme and is of the same order of magnitude as the supernatant DPNH-attacking system described above. It is not inhibited by 1 μgm/ml Antimycin-A. Fractionation of the supernatant revealed that some of the DPNH–cytochrome c reductase is localized on the microsomes, which were obtained by centrifuging at $25{,}000 \times g$ for 2 hours (International Refrigerated Centrifuge); this is also the case in liver homogenates (29).

It is possible that the activities of the supernatant may involve some small molecules, as well as enzymes, present in potato homogenates. Dialysis of the supernatant for 24 hours against distilled water at 4° C greatly diminishes its ability to carry out the reactions described above. Phenolic substances and quinones might participate in the redox reactions and could act as a carrier system, e.g. between DPNH and cytochrome c. To test the possibility that polyphenol oxidase might be involved, the supernatant was prepared in a medium containing 1×10^{-3} M phenylthiourea (PTU), a powerful inhibitor of this enzyme. This treatment resulted in the following inhibitions of supernatant activity: disappearance of characteristic DPNH absorption, 58 %; cytochrome c reduction without added substrate, 71 %; DPNH–cytochrome c reductase, 88 % (fig 7). Addition of PTU to the final reaction mixture was without effect. These results suggest that the SF-catalyzed reactions may involve, directly or indirectly, several different enzymes, one of which may be polyphenoloxidase.

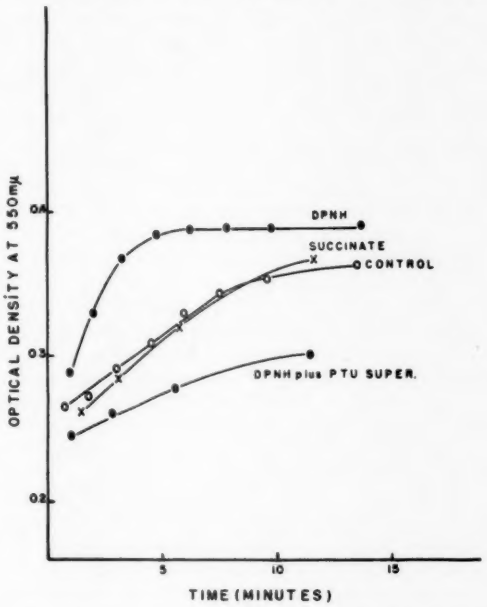


Fig. 7. The reduction of oxidized cytochrome c by the supernatant fraction. All cuvettes contained 2×10^{-5} M cytochrome c, 0.2 M sucrose, 0.01 M phosphate buffer (pH 7.0), and 0.001 M NaCN in a total volume of 3.0 ml. Open circle—Control, with 0.1 ml SF. Crosses—Succinate, with 0.1 ml SF and 0.015 M succinate. Crossed circle—DPNH, with 0.1 ml SF and 5×10^{-5} M DPNH. Solid circle—Contains 0.1 ml SF prepared in 1×10^{-3} M PTU, and 5×10^{-5} M DPNH.

Discussion

The results of the experiments with potato mitochondria are summarized diagrammatically in figure 8. The various components which were added experimentally to the particles are shown outside the limiting membrane, and the mitochondrial components are within. Arrows indicate the reaction sequences

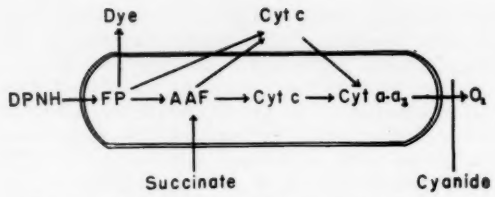


FIG. 8. Diagrammatic summary of electron transport pathways in potato mitochondrion.

suggested by the results. Since the particles are able to reduce dyes in the presence of Antimycin-A or cyanide it is assumed that DPNH reacts first with a diaphorase-type molecule. Diaphorase, which has been described in plants (14), is generally assumed to be a flavin-containing enzyme and is indicated by FP. The position of the Antimycin-A factor (AAF) is established by several lines of evidence: 1) Since DPNH oxidation is largely inhibited by AA, the factor must lie on the pathway between DPNH and oxygen; 2) Cytochrome c oxidation is insensitive to AA, so the factor is above the level of cytochrome c; 3) The almost complete AA-inhibition of succinic–cytochrome c reductase indicates that the factor lies between these two components; 4) The partial inhibition of DPNH-cytochrome c reductase by AA suggests a position between flavin and cytochrome c. The nature of this factor, which presumably corresponds to the BAL-sensitive Slater factor (25), is not definitely known, but it may be another heme-containing molecule or cytochrome (23). The major pathway between DPNH and added cytochrome c does not involve the factor. Since DPNH–cytochrome c reductase is a metal-containing flavoprotein which is closely related to diaphorase (24), its activity is indicated as an arrow from FP to cytochrome c. Finally, the particles possess an active cytochrome c oxidase, which is represented above as Cyt a–a_3.

Several points should be borne in mind when evaluating the pathways discussed above. The components shown in figure 8 do not represent all the carriers involved in the oxidation of DPNH and succinate. For example, succinic dehydrogenase itself includes flavin, heme, and non-heme iron, all of which may be involved in an electron conducting chain (1). The DPNH–cytochrome c reductase system which shunts around the AAF may well involve the participation of a cytochrome b (17). Spectrophotometric studies of Chance (3) and Lundegårdh (15) indicate that a cytochrome b-type component plays a major role in intracellular oxidations. The experiments reported here do not conclusively establish the fact that cytochrome c participates in the oxidation of added DPNH by the particles, although the sensitivity to AA and cyanide, as well as the presence of an active cytochrome c oxidase, is very suggestive. Finally, reconstruction of an electron transfer pathway from enzyme studies is made difficult by the structure of the mitochondrion itself. The presence of a limiting membrane and internal differentiation may affect the accessibility of externally added substrates to mitochondrial enzymes. For example, added cytochrome c permits an AA-insensitive oxidation of DPNH, whereas cytochrome c reduction within the mitochondria apparently proceeds via the factor. This raises the possibility that there are differing "internal" and "external" cytochrome c reductase molecules in the mitochondria (cf 13); alternatively, the effect might be due to contaminating microsomes. With respect to DPN-linked oxidations within the cell, it remains to be shown whether the AA-sensitive factor lies on the main pathway to oxygen. A system which could shunt around the factor to cytochrome c is present both in the particles and in the supernatant fraction; the great activity of the latter raises the possibility of an interaction between soluble and particulate components of the cytoplasm in respiration.

Since at least 70 % of the respiration of freshly-cut potato tissue is mediated by cytochrome oxidase (30), it is clear that the mitochondria play a central respiratory role in situ. Although the pathway of oxidation from substrate to the cytochrome system in intact cells has not been definitely established, the sequence proposed above could play this role. A comparison of the rates of the mitochondrial reactions suggests that DPNH oxidation is limited by the rate of reduction of cytochrome c within the particles; this is confirmed by the fact that the oxidation may be doubled by adding cytochrome c. This situation may well be the result of injury caused during the isolation of the particles.

The results reported here agree substantially with other attempts to reconstruct the pathway of terminal electron transfer in animal (3, 25) and plant (15) tissues. A comparison of relative enzyme rates reveals a high cytochrome oxidase activity in a wide variety of mitochondria: the ratio of cytochrome oxidase/DPNH–cytochrome c reductase is close to four in particles prepared from heart muscle (26), liver (9), housefly (22), and silkworm flight muscle (17), as well as potato tubers. The ratio of DPNH–cytochrome c reductase to succinic–cytochrome c reductase is 2/1 in mitochondria from potato and silkworm flight muscle (17) and 1/1 in heart sarcosomes (26). It is clear that the plant particles examined in this study show fundamental similarities to animal mitochondria.

In some respects the mitochondria isolated from various tissues show real differences. Whereas added cytochrome c does not affect the DPNH oxidase of heart muscle (25) and silkworm flight muscle (17), it does promote DPNH oxidation by liver (12), kidney (25), housefly (22), and silkworm midgut (17) mitochondria. In addition, the DPNH–cytochrome c reductase of heart muscle (25) and silkworm flight muscle (17) preparations is 95 % inhibited by Antimycin-A (or BAL), while that of kidney and liver (20) is inhibited only 20 to 30 %. The potato clearly resembles the later type of tissue, since its mitochondrial DPNH oxidase is stimulated by cytochrome

c and the DPNH–cytochrome c reductase can largely by-pass the AA-factor. The significance of these differences remains to be clarified. It is suggestive that heart and flight muscle are characterized by a classical c, b, a–a_3 cytochrome spectrum, whereas liver (29), silkworm midgut (17) and potato tissue (2) show a broad absorption band between 550 and 565 $m\mu$, indicating the presence of an additional cytochrome component.

The nature of the supernatant enzyme(s) reacting with DPNH is unknown. The possibility that the disappearance of the 340 $m\mu$ peak might be due to a splitting of the molecule seems unlikely since DPNase, which removes the nicotinic moiety, does not attack reduced DPN (10), and the pyrophosphatase split does not alter the absorption at 340 $m\mu$ (11). However, the reaction of DPNH with glyceraldehyde-3-phosphate-dehydrogenase is characterized by loss of the 340 $m\mu$ absorption band (19). A peroxidation of DPNH by H_2O_2 or organic peroxides in the supernatant is a possibility, since Dolin (4) has described a cyanide-insensitive DPNH-peroxidase from bacteria. The hydrogen of DPNH could be transferred enzymatically to some small molecule present in the supernatant; for example, plants have been shown to contain quinone reductase (31) and cystine reductase (21). The DPNH–cytochrome c reductase activity of the supernatant could be due either to a single flavin enzyme or to a more complex system involving other intermediate carriers. It is worth noting that free flavins alone, in the absence of protein, can catalyze the reaction between reduced pyridine nucleotide and cytochrome c (24). Active DPNH–cytochrome c reductase systems are apparently present in the mitochondria, the microsomes, and the soluble fraction, and their relative physiological importance remains to be determined.

Summary

An attempt has been made to reconstruct the pathway of electron transfer from reduced diphosphopyridine nucleotide (and succinate) to oxygen in cell free fractions prepared from potato tubers. Particles, assumed to be mitochondria, were isolated by differential centrifugation from a sucrose-phosphate homogenate of tuber tissue. The following enzymes and enzyme systems were assayed spectrophotometrically and their inhibitor sensitivities studied: cytochrome oxidase, DPNH oxidase, diaphorase, DPNH–cytochrome c reductase, and succinic–cytochrome c reductase. All of these enzymes are present on the mitochondria, and it is suggested that the oxidation of DPNH involves a successive electron transfer to flavoprotein, an Antimycin-A-sensitive factor, the cytochrome system, and oxygen. The isolated particles can also by-pass the AA-sensitive factor when DPNH is oxidized in the presence of added cytochrome c. The 10,000 × g supernatant fraction can oxidize DPNH rapidly and possesses an active DPNH–cytochrome c reductase system.

Since completion of this work, T. E. Humphreys and E. E. Conn (Arch. Biochem. Biophys., in press) have reported very similar results from a study of DPNH oxidation by lupine mitochondria. E. M. Martin and R. K. Morton (Nature 176: 113–114) have examined an Antimycin-insensitive DPNH–cytochrome c reductase activity of plant microsomes.

The writer wishes to thank Dr. K. V. Thimann for encouragement and advice throughout this investigation.

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THE EFFECT OF 2,4-DICHLOROPHENOXYACETIC ACID AND 2,3,5-TRIIODOBENZOIC ACID ON THE TRANSPORT OF INDOLEACETIC ACID[1]

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In the course of studies on the translocation of 2,4-dichlorophenoxyacetic acid (2,4-D) in plants (2, 3) it was deduced that, at high concentrations, 2,4-D interfered with the mechanism responsible for its own movement. Kuse (4) came to a similar conclusion for 2,3,5-triiodobenzoic acid (TIBA), when he found that auxin moving from the leaf did not pass the point where the TIBA was applied in a band of lanolin paste on the petiole. In this paper these deductions have been tested by direct methods.

The polar movement of indoleacetic acid (IAA) through excised sections of coleoptiles, stems, or petioles can be readily studied by the classical method of Went (6), wherein he placed donor agar blocks containing auxin, at the apical end of the section and blank receiver blocks at the basal end. The amounts transported to the basal block were assayed with the standard Avena test. By this method it was possible to test directly whether 2,4-D or TIBA exerts any influence on the mechanism responsible for the polar movement of IAA in excised sections of stems. The present work therefore comprises measurements of the translocation of IAA through sections of bean stems which had been pre-treated with 2,4-D or TIBA.

Experimental

Five-mm sections were taken from stems or petioles of 8- to 10-day-old bean seedlings (*Phaseolus vulgaris* var. Dwarf Red Kidney). These plants were raised in constant temperature light rooms at 19° C with their roots in water in small Erlenmeyer flasks. The actual transport experiments were conducted in a dark room using occasional red light. The sections were set upright on blank receiver blocks (1.5 % agar) and donor blocks containing a known amount of auxin were placed on the apical end. Small drops of water were placed between the agar and the cut surfaces to insure good contact during the translocation period. The blocks were $6 \times 6 \times 1.5$ mm so that when the receiver blocks were quartered the resulting blocklets were of the regular size for use in the Avena test. Each reading is the result of three sections set up in this manner to give twelve blocklets for testing. Usually the mean of two or more such readings are recorded below.

Curvatures Given by Mixtures of IAA and 2,4-D or TIBA: Since it was possible that some of the 2,4-D or TIBA used in the pre-treatments may get into the receiver blocks with the IAA, it was necessary first to know what influence these would have on the curvatures given by IAA in the Avena agar block test. Table I shows curvatures given for 2,4-D alone at 100, 10 and 1 mg/l and the curvatures given when these concentrations of 2,4-D were mixed with IAA at 0.1 mg/l. Small curvatures restricted to the top few mm were obtained with 2,4-D alone as was found by Avery et al (1). When IAA plus 2,4-D at 100 mg/l were tested, there was a depression in the amount of curvature produced compared with IAA alone at the same concentrations. On the other hand, when 2,4-D at 1 or 10 mg/l was given with the IAA, curvatures

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TABLE I

DEGREES OF CURVATURE IN THE AVENA AGAR TEST WITH MIXTURES OF IAA AND 2,4-D

CONCENTRATION, MG/L		DEGREES CURVATURE
IAA	2,4-D	
0.1	...	25.2
0.1	100	12.5
0.1	10	31.8
0.1	1	40.7
...	100	6.0
...	10	7.5
...	1	8.7

larger than for IAA alone were obtained. A similar behavior at high and low concentrations was found by Thimann and Bonner (5) for TIBA when mixed with IAA in the Avena test. TIBA alone was unable to produce any curvatures. Since it will be shown below that only a small fraction of the applied auxin reaches the receiver blocks, it follows that the concentration of 2,4-D or TIBA attained therein would not decrease the curvatures induced by IAA in the Avena test but if anything they would increase the response.

TRANSPORT OF IAA THROUGH SECTIONS FROM VARIOUS PARTS OF BEAN SEEDLINGS: It was next necessary to determine the amount of basipetal transport of IAA through 5-mm sections taken from various parts of bean seedlings. The donor blocks contained IAA at 10 mg/l and transport lasted 3 hours. Table II shows the average curvatures produced by receiver blocks. A concentration of 25 μgm/l in the receiver blocks would have yielded curvatures of about 15° so that only very small amounts of IAA moved through sections of the roots since curvatures of less than 5° were obtained. On the other hand, enough auxin moved through the sections of the stem to cause curvatures close to the maximum produced by 250 μgm/l.

TRANSPORT OF IAA AS A FUNCTION OF THE AMOUNT IN THE DONOR BLOCKS: Table III shows results obtained when transport through sections of the hypocotyl was studied as a function of the amount of IAA in the donor blocks. These data were compiled from a large number of trials. Some of the curvatures produced by the receiver blocks were as large as the maximum obtainable in the test so that the actual amounts had to be determined after making the appropriate dilutions. Although the percentage of IAA moving through these sections decreased as the amount applied was raised, the actual amounts moving through increased. This agrees with the results of Went (7) who found that he had to raise the level of IAA in the donor block to 1000 mg/l before the amount that moved through coleoptile sections fell off. In all further trials, donor blocks containing IAA at 10 mg/l were used since this concentration was without harmful effects and was high enough so that large curvatures were obtained.

TABLE II

TRANSPORT OF IAA IN 3 HOURS IN AN APEX TO BASE DIRECTION THROUGH 5-MM SECTIONS FROM VARIOUS PARTS OF BEAN SEEDLINGS. DONOR BLOCKS CONTAINED 10 MG/L. MEANS OF FROM 2 TO 4 EXPERIMENTS

SECTIONS FROM	DEGREES CURVATURE
Petiole	30.6
Epicotyl	31.3
Hypocotyl—7 mm from top	27.3
Hypocotyl—10 mm from bottom	24.7
Root-stem juncture	4.7
Root—2.5 mm from top	3.0
Root—7.5 mm from top	3.7
Controls—250 μgm/l IAA	ca 30.0
" —25 μgm/l IAA	ca 15.0

TABLE III

TRANSPORT OF VARIOUS AMOUNTS OF IAA THROUGH 5-MM SECTIONS OF HYPOCOTYLS OF BEAN SEEDLINGS DURING A PERIOD OF 3 HOURS. MEANS OF FROM 2 TO 5 EXPERIMENTS

CONC OF IAA IN DONOR BLOCKS, MG/L	CONC OF IAA IN RECEIVER BLOCKS, MG/L	PERCENT TRANSPORTED
100	0.15	0.15
10	0.025	0.25
1	0.01	1.00
0	0.0	...

PRE-TREATMENT OF STEM SECTIONS WITH 2,4-D: Next, the ability to transport IAA was measured after the stem sections had been pre-treated with 2,4-D. For this pre-treatment, the sections were placed upright on wet filter paper and agar blocks containing 2,4-D at 0, 10 and 100 mg/l were placed on the apical ends. After 4 hours, these blocks were replaced with plain agar blocks and the pre-treatment period continued for 3 more hours. The sections were then placed on blank receiver blocks, and the donor blocks containing IAA at 0 and 10 mg/l were placed on the apical ends. The amount of IAA translocated in 2 hours was then measured.

The results in table IV show that when the sections were pre-treated with 2,4-D at 10 mg/l there was a reduction in the curvatures produced by the receiver blocks, and following pre-treatment with 100 mg/l there was a very marked inhibition. Actually, the curvatures obtained for the latter were no different from the controls for which the donor blocks contained no IAA. This response could not be attributed to an inhibitory influence on the Avena curvature by 2,4-D which may have moved into the receiver blocks.

TABLE IV

TRANSPORT OF IAA IN 2 HOURS THROUGH 5-MM SECTIONS OF BEAN HYPOCOTYLS PRE-TREATED WITH 2,4-D

PRE-TREATMENT	TRANSPORT OF	DEGREES CURVATURE
Blank	IAA 10 mg/l	20.8
2,4-D 10 mg/l	Blank	1.4
2,4-D 10 mg/l	IAA 10 mg/l	15.5
Blank	IAA 10 mg/l	24.8
2,4-D 100 mg/l	Blank	6.8
2,4-D 100 mg/l	IAA 10 mg/l	7.1

TABLE V

TRANSPORT OF IAA IN 2 HOURS THROUGH SECTIONS REMOVED FROM THE EPICOTYL AND PETIOLE OF BEAN SEEDLINGS, THE PRIMARY LEAVES OF WHICH HAD BEEN TREATED WITH 50 μGM OF 2,4-D OR TIBA 24 HOURS PREVIOUSLY. DONOR BLOCKS CONTAINED 10 MG/L IAA. MEAN OF 2 EXPERIMENTS

TREATMENT	DEGREES CURVATURE PRODUCED BY RECEIVER BLOCKS	
	EPICOTYL SECTIONS	PETIOLE SECTIONS
No pre-treatment	31.3	30.6
Pre-treated with 2,4-D ..	7.3	14.4
Pre-treated with TIBA ..	2.3	...

The maximum amount of 2,4-D one could reasonably expect in the receiver block would not give a concentration of more than 1 to 10 mg/l since in table II only 1 % of the auxin was transported. These concentrations of 2,4-D would give a synergistic response with the IAA rather than an inhibitory one (table I). It is concluded, therefore, that smaller amounts of IAA are transported when the sections have been pretreated with 2,4-D.

PRE-TREATMENT OF INTACT SEEDLINGS WITH 2,4-D OR TIBA: The applicability of these results to whole plants is of interest in connection with herbicide work. The transport of IAA was, therefore, measured in sections of stems taken from bean seedlings which had previously received a foliage application of 2,4-D or TIBA. Fifty micrograms were applied to one of the primary leaves of the seedlings in the light, and at 24 hours 5-mm sections were removed from the epicotyl and petiole. The sections were set on moist filter paper for two hours to allow any auxin already present to be transported out. After this time, their ability to transport IAA was tested as before. Analysis made in other studies (3) showed that 2,4-D is transported from the leaf to the epicotyl and that these 5-mm sections would contain about 0.5 μgm of 2,4-D under the above conditions. If all of this 2,4-D moved into the receiver blocks, which is extremely unlikely, the final concentration would be 15 mg/l. This concentration would tend to increase the response given by IAA rather than to depress it.

The Avena curvatures produced by the receiver blocks after a two-hour translocation period are recorded in table V. The donor blocks contained 0 and 10 mg/l. Quite substantial amounts were transported through both epicotyl and petiole sections of untreated plants, but much smaller amounts moved through sections taken from seedlings which had been previously treated with 2,4-D or TIBA. To this extent the data for TIBA confirm Kuse's conclusion previously mentioned.

It seems clear, therefore, that the mechanism which is responsible for the transport of IAA through excised sections of bean stems is disrupted when 2,4-D or TIBA is applied to the foliage. If this transport mechanism operates in intact plants, it seems certain that when these compounds are applied to the leaves or stems they will interfere very greatly with auxin transport.

SUMMARY

Transport of IAA through excised sections of stems and roots from bean seedlings was studied. Pre-treatment with 2,4-D or TIBA inhibited subsequent translocation of IAA.

The author wishes to express his appreciation to Dr. K. V. Thimann for his interest and assistance in this work.

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WATER ABSORPTION BY NEEDLES OF PONDEROSA PINE SEEDLINGS AND ITS INTERNAL REDISTRIBUTION[1,2]

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In 1727 the first published report dealing with the uptake of dew by the plant leaf appeared. At that time Stephen Hales reported that ". . . perspiration on a dry warm night, without any sensible dew, was about three ounces, but when any sensible, though small dew, then the perspiration was nothing; and when a large dew, or some little rain in the night, the plant and pot was increased in weight two or three ounces" (20).

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[2] Work carried on under University of California Agricultural Experiment Station Research Project 1577.

During the following 225 years a number of papers have appeared dealing with one or more aspects of the relationship between plant growth and natural occurring leaf surface water such as dew. Unfortunately, much of this work is difficult to evaluate since it was of an ecological nature and the line between fact and speculation not clearly drawn. However, it is readily apparent that 4 main concepts regarding the importance of leaf surface water have dominated the literature. These are: first, that leaf surface water is unimportant or even harmful (15, 37, 41, 47, 55, 58, 59, 60); second, that leaf surface water is important because it reduces transpirational loss (11, 30, 32, 42, 45, 48); third, that leaf surface water is important because of the amount of water it furnished the plant per se (2, 12, 13, 16, 17, 21, 23, 28, 31, 33, 35, 36, 38, 39, 40, 43, 44, 46, 50, 51, 54, 61); and fourth, that leaf surface water is important because unlike water taken in through the roots, it is free of salts and therefore tends to lower the salt concentration within the leaf (1, 24, 25, 29, 49, 62).

Related to the question of leaf surface water and its utilization by the plant is the process of water redistribution within the plant itself. The reports by J. F. Breazeale and his co-workers (3, 4, 34) present some of the first work on this water redistribution problem. Their work indicated that the roots of corn and tomato plants could absorb water from soil above the wilting point and then "pump" it out again through other roots as they grew into soil which was below the wilting point. Hendrickson and Veihmeyer (23) questioned these results when they were unable to repeat this phenomenon with sunflower and beans.

However, about the same time Wadsworth (57) carried out a series of experiments with sugar cane which supported Breazeale's conclusions regarding the outward movement of water through the roots into the soil. He found that either light rain or spray from a garden hose falling upon the leaves of a potted sugar cane plant increased the moisture content of the soil surrounding the roots. Since the soil surface was sealed off by an asphalt emulsion, he concluded that the increased moisture content of the soil was the result of water moving back through the leaves into the plant and then out through the roots into the soil.

Although Brierley (10) did not observe any build-up in soil moisture when wilted raspberry plants were exposed to a water spray, he did note that when water spray was applied to one of a pair of wilted canes the leaves on both canes became turgid again. This strongly suggested that water applied to the leaves of one wilted cane moved in and downward and then upwards into the other cane.

Recently E. L. Breazeale and his co-workers (5, 6, 7, 8, 9) reopened the question of the redistribution in the plant of water moving in through the leaf surfaces. They reported that both tomato and corn plants could move water back into the soil when their tops were exposed to a continuous spray. In addition ,they found that soil was not necessary. Water could still be "pumped" back out through roots even when they were sealed into an empty flask.

Haines (18, 19) enlarged upon the empty flask experiment and obtained a similar accumulation of water in the flask with either a tomato plant or a cotton wick. However, if the temperature in and around the flask was held constant, water failed to accumulate. He further found that when the top of a tomato plant was cut off, the stem inserted into a capillary tube filled with water, and the leaves enclosed in a fogging chamber, the meniscus in the tube failed to move either up or down. Such data certainly indicate that water accumulation in the empty flask was due to a vapor pressure gradient and not to any active secretion of water by either the leaves or the roots.

Hohn (26) unlike Haines, was unable to repeat any of Breazeale's results. However, he did find that liquid water is more easily absorbed than water vapor by the shoot part of the plant. He also showed that water could be evaporated from an object even though the surrounding atmosphere was apparently saturated with water.

Janes (27) in agreement with Hohn, found that plants could continue to lose water through their leaves even in a continuous fog-spray. In his experiments he found that six-inch tomato plants, when sealed into flasks filled with soil close to field capacity and placed in a fog chamber, would reduce the moisture content of this soil to near the wilting point during an eighteen-day period.

In a recent paper Stone and Fowells (53) reported that ponderosa pine seedlings can reduce the soil moisture below the ultimate wilting point of the sunflower and that under this high moisture stress, artificial dew at night prolongs the life of the seedlings. It is not apparent from their data how this was accomplished. One possibility, in line with the redistribution concept supported by Breazeale and others, is that under such drought conditions water is absorbed through the needles and then redistributed throughout the plant. On the other hand redistribution might not be involved. Instead, it could be the effect of nightly resaturation of the needle tissue which in turn could reduce the over all transpirational loss.

This paper reports on experiments designed to evaluate the first suggestion, namely the existence of a system capable of internal redistribution of water absorbed by the leaves (needles). Intake of water from a continuous mist spray and its subsequent movement in seedlings rooted in soil and in seedlings whose roots were sealed into empty flasks was studied.

Method of Study

Two-year-old ponderosa pine seedlings from the U. S. Forest Service nursery at Oakdale, California were used throughout these studies. When these seedlings were received in Berkeley they were immediately placed in cold storage and held at 5° C

until used. The seedlings were in excellent condition with well developed top and root systems.

Roots Sealed in Empty Flasks: In the first experiment the seedlings were removed from storage to the greenhouse, where the dirt and peat moss in which they were stored were washed from the roots. Then roots were shaken free of water and lightly blotted with paper toweling. Next the roots of each seedling were inserted into the top of a 2-liter suction flask. A split single-hole rubber stopper was carefully fitted around the stem of the seedling and inserted into the mouth of the flask. The stopper was pushed in very tightly by the use of a clamp system, and checked for leaks. The flask with its contained seedling was then turned upside down in the open greenhouse (fig 1). Water that accumulated in the flask was drained off at regular intervals and measured.

The second experiment was the same as the first except that the flasks were not turned upside down in the open greenhouse; instead they were placed in a constant temperature water bath.

In the third and fourth group of experiments dead seedlings were used in place of the live ones. One group of these seedlings was killed by desiccation before the roots were sealed into the flasks. The other group was killed by inserting the tops into a beaker of boiling water after the roots had been sealed into the flasks.

The last experiment of this series consisted of recording relative humidities inside the flasks when they were placed in the control temperature bath and when they were turned upside down under the mist spray in the greenhouse. This was accomplished with an Aminco Dunmore humidity sensing unit sealed into the flask in place of the seedling (52). Before these flasks were sealed, 25 ml of water were added to wet the inside surface of the flask.

Roots Sealed in Soil at the Wilting Point: In January, 20 seedlings were removed from storage and planted singly in gallon cans of forest soil. This soil was from a natural stand of pine and contained the normal complement of mycorrhiza and other micro-organisms. The ultimate wilting point as determined with sunflowers was 13.8 %. The plants were watered regularly in the greenhouse. By July their root systems fully occupied the lower two thirds of the can.

At this time 15 cans were selected for the experiment on the basis of the uniformity of the seedlings. Five sunflower seeds were then planted around each pine seedling. After germination they were thinned to 3 per can. Because of low fertility of the soil an occasional watering with Hoagland's solution increased to 5 times its regular strength was required for good sunflower growth.

When all the sunflowers had developed at least 12 fully expanded leaves, watering was discontinued for the remainder of the experiment. By this time the roots of the sunflower had completely permeated the soil mass in the cans.

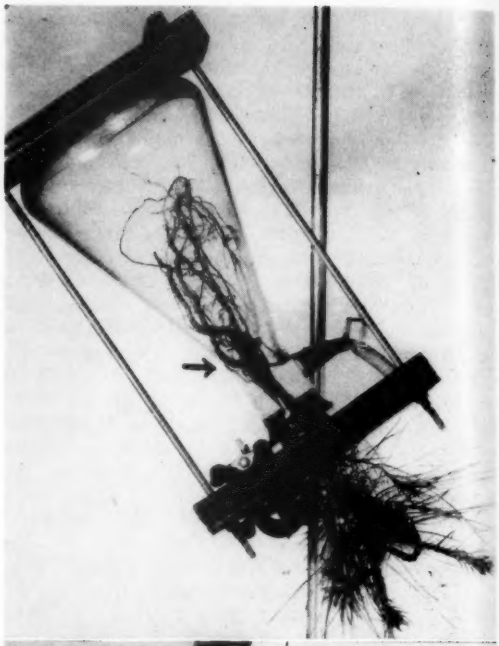

Fig. 1 (*top*). Seedling after 6-day exposure to a continuous mist spray. Water accumulation indicated by arrow.

Fig. 2 (*bottom*). Canned seedlings in rack ready for exposure to the mist spray. Each seedling was sealed in a can with a plastic envelope after sunflowers which reduced the soil moisture to the wilting point had been clipped off.

After watering was discontinued, the moisture content of the entire soil mass was gradually reduced. First to the permanent wilting point at which time the sunflowers permanently wilted and

then to the ultimate wilting point at which time they died (14, 56).

All of the sunflowers, but none of the pines, had died within 15 days after watering was discontinued. Soil moisture was then determined as it was dug from 5 of these cans. In the 10 remaining cans the dead sunflowers were clipped off at the ground level and each can was sealed with a plastic envelope through which the pine seedling protruded. A water-tight seal around the stem was accomplished with several turns of a fine copper wire and a heavy wrapping of electrical Scotch tape. Drain holes in the cans were sealed with hot paraffin.

Five of these sealed cans were set aside on a bench in the greenhouse as a control. The remaining 5 cans were laid on their sides in a rack resembling a multiholed guillotine minus the blade (fig 2). It was then possible to arrange a fog-spray nozzle so that a fine mist spray landed on the pine needles, but not on the cans. Since the tops of the cans tilted slightly downward the absence of leaks was assured. To prevent any differential heating of the cans and the accompanying redistribution of soil moisture within the cans, 5 cm of rock wool insulation and a sheet of aluminum foil was laid over the cans.

The tops of these seedlings were exposed to a continuous spray for a period of 6 months, after which time, the experiment was discontinued. The roots during this time were in soil whose moisture content at the initiation of the experiment had been reduced to the ultimate wilting point of the sunflower.

At the end of this 6-month period the cans and seedlings whose tops still appeared alive and in good condition were removed to the laboratory where the soil moisture was determined.

Results

Roots Sealed in Empty Flasks: Water moved backward through the needles of the live seedlings and out through the roots provided the flasks were allowed to heat-up during the day. The same was true when the live seedlings were replaced by seedlings whose tops had been killed by boiling water. However, when the seedlings were killed by desiccation, water failed to accumulate. It also failed to accumulate when the flask temperatures were held constant by immersion in a water bath (table I).

A marked difference in the relative humidity was observed in flasks whose temperatures were allowed to fluctuate as opposed to those whose temperatures were held constant. Under constant temperature conditions the relative humidity remained at 100 %. Under fluctuating temperatures it fell below 100 %. On sunny days for example, relative humidities as low as 85 % were recorded (table II).

Roots Sealed in Soil at the Wilting Point: During the period of the experiment the cans did not change in weight. If any reverse movement of water back into the soil did occur, an equal amount must have been lost during this same period of time. Furthermore, there was no change in the soil moisture distribution within the can. The average moisture content at each sampled depth within the cans was the same at the end of the experiment as it was at the beginning (table III).

The seedlings in the 5 cans which were set aside on a bench in the greenhouse as a control were all dead within a month. The needles on the seedlings placed under the continuous spray were still green and appeared healthy when removed at the end of 6 months. However, when the seedlings were removed from the soil at this time and examined, succulent white root tips which are normal on a healthy actively growing seedling were absent. Furthermore when these same seedlings were placed with their roots in

Table I

Milliliters of Water (Accumulative Totals) Collected from Flasks When Seedling Tops Were Exposed to a Continuous Mist Spray

Elapsed time	Live seedlings *				Top of seedlings killed by boiling water **			
days	*ml*				*ml*			
1	1.0	3.0	0.5	1.5	0.5	0.5	1.5	2.0
4	5.0	5.5	1.5	4.5	1.5	2.0	2.0	3.5
7	8.5	7.5	2.5	6.5	2.0	3.0	3.5	4.0
13	13.5	10.5	4.5	10.0	4.0	4.5	7.0	5.5
19	21.0	11.5	6.0	13.0	5.0	6.0	8.0	5.5
25	29.5	13.5	8.0	16.5	7.0	6.0	9.5	5.5
31	45.0	14.5	9.0	20.5	8.0	6.0	9.5	5.5

Flask temperatures allowed to fluctuate.
* When flask temperature was held constant, water failed to accumulate.
** When seedlings were killed by desiccation, water failed to accumulate.

Table II

Relative Humidity in the Flasks *

Time	Sky condition	Temp inside flask °C	Relative humidity inside flask ** %
Flasks in constant temperature bath			
1100	Cloudy	16	99
1345	Cloudy	16	99
1430	Sunny	16	99
1500	Sunny	16	99
1530	Sunny	16	98
Flasks not in constant temperature bath			
0900	C'oudy	15	98
1000	Cloudy	21	93
1230	Sunny	24	90
1235	Sunny	25	85
1500	Sunny	28	83

* Flasks were empty except for 25 ml of water.
** Relative humidity readings, with the Aminco-Dunmore sensing units, above 98 % are unreliable. They represent anything from 98 to 100 % relative humidity and must be treated accordingly.

TABLE III

SOIL MOISTURE CHANGE DURING EXPOSURE OF SEEDLING TOPS TO A CONTINUOUS MIST SPRAY*

DEPTH IN INCHES	% MOISTURE INITIALLY (AFTER SUNFLOWERS HAD DIED)		% MOISTURE 6 MOS LATER	
	AV.**	RANGE	AV.**	RANGE
0–2	11.3	10.0–12.7	12.0	10.4–13.3
2–4	13.0	11.5–14.0	12.9	11.7–13.7
4–5	12.9	12.3–14.1	13.4	12.4–14.2
5–6	13.0	12.7–13.5	13.2	11.5–14.0

* Soil moisture change during the 6-mo period was not statistically significant at any depth as determined with the t-test.

** Average of 5 cans.

water their needles changed from a dark healthy green to a bleached shriveled condition in less than 24 hours.

DISCUSSION

In view of contrasting reports and different interpretations of the empty-flask type of experiment the above results with pine seedlings are of particular interest. Pine is apparently no different from the tomato plant in its ability to transport water from a saturated atmosphere back through its roots into an empty flask (5, 6, 7, 8, 18, 19).

The water movement in this completely artificial system is apparently the result of a vapor pressure gradient extending from an area of 100 % relative humidity to one of a lower relative humidity, with the plant in between. Basically this is the wick explanation advanced by Haines (8, 19). When this vapor pressure gradient does not exist, the mechanism for a backward movement of water through the plant apparently does not exist. Thus water would not be expected to accumulate when the flasks are kept at a constant temperature and the relative humidity inside remains at 100 %. On the other hand when the flasks are removed from the water baths their temperatures fluctuate, relative humidities below 100 % develop, and a vapor pressure gradient results which in turn causes water to move into the needles and out of the roots.

As Hohn (26) has pointed out it is possible for evaporation to take place from an object in a completely saturated atmosphere provided its temperature is higher than the surrounding air. However, in these experiments when the flasks were submerged in a constant temperature bath, the roots were not exposed to radiant energy. The only other way the root temperatures could be raised above the surrounding air temperature would be through metabolic activity. However, metabolic activity is too low to be of significance in affecting the air temperature and thus the vapor pressure deficit. Therefore, for all practical purposes the root and the surrounding air must have been at the same temperature.

In these studies the backward movement of water through the plant was always associated with a drop in the relative humidity in the flask and the consequent vapor pressure gradient described above. Even when the seedling was dead water still moved backward, provided it had not been allowed to dry out first. In effect the seedling acted like the cotton wick used by Haines (18, 19). As water was lost by evaporation from the root surface it was replaced by water further back in the root tissue and so on until finally water in the needles moved inward toward the roots. Water from the mist spray continually resaturated the needles thereby supplying a continuous stream of water back through the plant.

When the seedling was killed by immersing the top in boiling water its tissues were still hydrated and acted as an effective wick. The smaller volumes of water accumulated by these seedlings may have resulted from the collapse and plugging of some of the tissue.

A dried shriveled pine seedling is obviously not a good wick. Water movement through such a system would be primarily through the cell walls. Nevertheless, when such a seedling was used to replace the living seedling the same vapor pressure gradient still existed and water should still be able to move inward. However, the rate would be extremely slow. Apparently in these studies not enough time was allowed for water to move inward and accumulate.

If the same principle works in a natural system where the plant roots are surrounded by soil, a similar vapor pressure gradient must be present if water is to move out into the soil. A vapor pressure gradient apparently does not exist in soil above the permanent wilting point since at this point the relative humidity in the soil is very close to 100 %. For example, at 20° C and an absorption force of 15 atmospheres, the relative humidity in the soil would be 99 %. Even at the ultimate wilting point, a negligible vapor pressure gradient would exist. This would explain why moisture did not move back through the seedlings in sufficient quantities to be detected by the methods used when their roots were in soil at the wilting point and their tops were in a continuous mist spray.

The experimental data presented above do not reflect upon the credibility of experiments published elsewhere which reported that mist-spray at night (artificial dew) prolonged the life of ponderosa pine seedlings after the soil had reached the wilting point (53). However, the data presented do indicate that prolonged survival under such circumstances is not due to water being "pumped" back into the soil.

As now visualized, the pine seedling, under conditions of soil drought, is able to live longer with spray at night (artificial dew) because of a resaturation of the needle tissue and a concomitant reduction in the amount of moisture removed from the root system. The root system can apparently live for several weeks under these conditions. Under a continuous spray with soil at the wilting point, the roots eventually die while the top remains alive much the same as a branch stuck in a glass of water.

Summary

The tops of two-year-old ponderosa pine seedlings were exposed to a continuous mist-spray after their roots had been sealed into either empty flasks or cans of soil. Under these conditions water moved back through the plant and out through the roots only when the temperature in the empty flasks was allowed to fluctuate. When the temperature was held constant this did not occur. Those plants whose roots had been sealed in soil also failed to show any backward movement of water out through their roots.

From these experiments it would appear that the backward movement of water through the plant is the result of a vapor pressure gradient and is not due to any "active" secretive force within the plant itself.

The prolonged survival of pine seedling in soil at the wilting point when the tops receive dew at night, as reported elsewhere, cannot be explained on the basis of water movement back out through the roots into the surrounding soil. As now visualized the seedling under such conditions is able to live longer because of a resaturation of the needle tissue and a concomitant reduction in the amount of moisture removed from the root system.

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METABOLIC ASPECTS OF NEUROSPORA ACTIVATION AND GERMINATION[1,2]

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The discovery by Shear and Dodge (19) that the dormancy of ascospores of Neurospora could be broken by heat-treatment led to the studies of Goddard (7, 8) who concluded that the lack of the enzyme carboxylase was the metabolic block that was overcome by activation. The presence of the enzyme in activated cells was inferred from experiments showing that pyruvic acid could reverse the toxicity of fluoride toward anaerobic CO_2 production. On the other hand, dormant cells were considered to lack carboxylase because exogenously added pyruvate failed to stimulate their fermentative release of CO_2. It was to critically examine these conclusions and to further delineate the steps involved in the activation process that the following experiments were undertaken.

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Materials and Methods

Ascospores of *Neurospora tetrasperma* were used

as produced from a cross of strains 374 and 377 obtained through the kindness of Dr. B. O. Dodge. After being harvested by the techniques described by Goddard (7), the ascospores were suspended in 0.8 % ethylene diamine tetraacetic acid (variously called versene or EDTA) and shaken at least 18 hours. This treatment served to prevent clumping and to kill contaminant conidia, without any adverse effect upon the ascospores. Before being used, the ascospores were rinsed free of the versene by centrifugation in 5 changes of distilled water.

Dry weights were determined directly after the spores were dried to constant weight at 105° C, or indirectly by multiplying hematocrit volumes by 0.36 (7).

Heat activation was accomplished by placing ascospores suspended in water or buffer in a water bath held at 58° C for 15 minutes. Since only heat-activation was used in the experiments to be reported, the aging phenomenon important in the response of ascospores to chemical activators could be disregarded. However, in no case were spores used that were harvested more than a year previous to use.

Gas exchange was measured with the Warburg apparatus using standard techniques. A shaking rate of 120 oscillations per minute at a temperature of 26° C was used throughout. Anaerobic conditions, when used, were effected by flushing with 100 % nitrogen or with 95 % nitrogen and 5 % carbon dioxide. In all cases, contaminant oxygen was removed by passage of the gas mixture through freshly prepared alkaline pyrogallol or over heated copper which had previously been reduced with hydrogen gas.

Ethanol determinations were performed using alcohol dehydrogenase and measuring the reduction of diphosphopyridine nucleotide (DPN) at 340 mμ in a model DU Beckman spectrophotometer, as described by Racker (17) and Bonnichsen and Theorell (1). The twice crystallized enzyme was obtained from the Sigma Chemical Company, and the DPN was a product of the Pabst Research Laboratories and was approximately 75 % pure.

Alcohol dehydrogenase activity was determined by incubating a spore extract in 0.1 M ethanol and measuring the reduction of DPN as described above. The spore extract was obtained by grinding 250 mg of whole ascospores with a glass tissue homogenizer in 10 ml of 0.01 M pyrophosphate buffer at pH 8.8. Centrifugation at 1000 × g was then sufficient to permit the removal of cell debris. All operations were carried out at 4° C.

Apocarboxylase and cocarboxylase were determined by the methods of Westenbrink and Steyn-Parvé (27). One hundred mg of spores were suspended in 1 ml of 0.01 M phosphate buffer at pH 6.2 and activated or used in the dormant condition, after which they were ground as above. Each Warburg vessel contained 0.8 ml of extract and 0.1 ml of cocarboxylase (100 μgm per ml) obtained from Nutritional Biochemicals, Incorporated. At zero time, 0.2 ml of 1 M sodium pyruvate in 0.5 M phosphate buffer at pH 6.2 was tipped from the side-arm and the CO_2 determined under nitrogen. Cocarboxylase was also determined manometrically using yeast apocarboxylase prepared according to Green et al (10). One ml of a thiamin solution containing 2.0 mg/ml was added to 400 mg of ascospores in 2 ml of distilled water. The addition of thiamin was necessary in order to preclude the dephosphorylation of cocarboxylase (27). The spores were ground in the cold and the supernatant recovered after centrifugation at 1000 × g. An aliquot of the supernatant was used for nitrogen determinations and 2 ml of the remainder were used for the cocarboxylase assay. To accomplish this, 1 ml of 0.050 M HCl was added as a protein precipitant and the container suspended in boiling water for 1 minute whereupon the extract was cleared by centrifugation and immediately adjusted to pH 6.2 with dilute KOH. The Warburg flasks contained 0.7 ml of the extract, 0.2 ml apocarboxylase and 0.2 ml of 1 M sodium pyruvate at pH 6.2 in the side-arm. Controls were run with 0.7 ml distilled water instead of the extract. Under these conditions, the rates of CO_2 evolution were linear for the entire duration of the experiment (50 minutes).

Pyruvic acid was determined by the method of Friedemann and Haugen (6) and readings were made at 420 and 540 mμ. Lithium pyruvate was used as the standard and concentrations in spore extracts were based upon total nitrogen.

Acetaldehyde analyses were carried out by the method of Stotz (20) and Westerfeld (28). A known volume of ascospores, as determined in a hematocrit tube, was aerated with a slow stream of moist air and the effluent containing the acetaldehyde was collected in cold freshly prepared 2 % sodium bisulfite. The bisulfite solution was then used directly for acetaldehyde determinations by the above method using paraldehyde (4 μgm per ml) as a standard.

Cytochrome oxidase was extracted by grinding a weighed sample of the ascospores in a solution of cold 0.5 % sucrose and 0.67 M phosphate buffer, pH 7.4, by means of a tissue homogenizer. This material was then centrifuged at 100 × g for 15 minutes and the residue discarded. All operations were performed in the cold. An aliquot of the supernatant was added to Warburg vessels and the oxygen uptake was measured in the presence of excess cytochrome C (Sigma) as the substrate and hydroquinone as the reducer (9, 10).

Nitrogen determinations of the extracts used for chemical and enzymatic analysis were made by means of a modified Nesslerization procedure after digestion with 1:1 concentrated sulfuric acid (13).

Extracts were prepared for paper chromatography by grinding 500 mg of spores in a jacketed Waring blendor cup containing approximately 20 ml water and enough acid-washed glass beads to make a thick slurry. About 4 ml of 15 % perchloric acid were

then added and the precipitate removed by filtration. The residual perchlorate was removed by filtration in the cold after the addition of enough 10 % KOH to bring the pH to neutrality. The filtrate was then brought to pH 3.0 with HCl and extracted to completion with ether for 18 hours in a liquid-liquid extractor. Evaporation of the ether extract to dryness was accomplished in a vacuum desiccator after which this material, in a few drops of water, was spotted on Whatman #1 paper. Two solvent systems were used in the analysis of organic acids. The first was absolute ethanol, water and concentrated ammonia (80:15:5) (v/v) run as an ascending liquid phase for 10 hours at room temperature. After being dried for two hours it was run perpendicularly to the initial direction in a descending manner. The water poor phase of a solvent system consisting of mesityl oxide, formic acid and water 75:36:75) (v/v) was used and the chromatogram was run for 10 hours. Again the chromatogram was dried for a minimum of 2 hours after which it was sprayed with a solution of 0.1 *N* $AgNO_3$ and 0.1 *N* NH_4OH in equal amounts. Pink, gray, yellow and white spots appeared on a tan background after 4 hours at room temperature in subdued light (2). By this means the color of the spots, as well as the R_f, aids in the presumptive identification of the acids.

Chromatograms of the keto acids were prepared by running the phenylhydrazone derivatives as recommended by Cavallini et al (3), and Hockenhull et al (11). However, it was found necessary to remove interfering materials by treating the extract with bisulfite as recommended by Kvamme and Hellman (14) in order that the acids run as described.

In order to clarify the subsequent discussion of results, the terminology of the different physiological stages of the ascospore has been used as suggested by Goddard (8):

dormant—those cells that have not had any treatment administered that would induce them to germinate.

activated—those cells that have been treated so that under the appropriate conditions of incubation they will germinate.

germinated—those cells that have been activated and incubated until germ tubes have protruded from the ascospore.

vegetative—that phase of the organism's development after germination and before the production of reproductive bodies.

Results

Apocarboxylase Content of Ascospores: Extracts from 100-mg lots of ascospores were prepared as described in the section on methods. These spores had previously been heat-activated in 4 ml of distilled water and were incubated for varying lengths of time by being aerated with a slow air stream. Dormant control samples were also run in the same way and the results of these experiments summarized in table I. Although there does not seem to be significant variation in the apocarboxylase activity of activated ascospores, dormant ascospores have somewhat less activity than do activated ones. In fact, heat activation alone (0-hour samples) induced a 50 to 60 % increase in activity.

Table I

Apocarboxylase Activity of Dormant and Activated Ascospores of Neurospora tetrasperma

Time after activation	Apocarboxylase activity
hrs	*μl CO_2/50 min × mg nitrogen*
Dormant controls	616 ± 37.9
0	1013 ± 67.2
1	894 ± 14.0
2	920 ± 36.1
3	895 ± 40.0

All figures represent the mean of three determinations and the standard deviations calculated from these. The rate of evolution of carbon dioxide was calculated between 5 and 15 minutes after the tipping of pyruvate.

Since furfural has been shown to activate dormant ascospores (5, 24), its effect upon apocarboxylase activity was studied. Extracts from dormant ascospores were prepared as before with the exception that 1×10^{-3} M furfural was added before the measurement of activity was begun. The results presented in table II show only a small effect of furfural in depressing the carboxylase activity of Neurospora ascospore extracts.

The Michaelis constant (K_m) for the apocarboxylase of the ascospores was then calculated by measurement of the activity obtained from extracts of 200 mg of ascospores in the presence of different concentrations of pyruvate. The results are plotted in figure 1 and show that the K_m obtained for the enzyme in dormant ascospores is 4.2×10^{-2} M. However, the curve obtained for the activated ascospores seems to consist of at least two components. At the lower substrate concentrations the K_m approaches that obtained with extracts of dormant ascospores, but at the higher concentrations the K_m decreased to approximately 0.9×10^{-2} M. Although the absolute value of this latter constant has been derived from only two points in figure 1, repeated experiments have always disclosed this break in the curve.

The Cocarboxylase (Diphosphothiamin) Content of Ascospores: To complete the studies of the

Table II

Effect of Furfural upon the Carboxylase of Dormant Neurospora Ascospores

Treatment	Apocarboxylase activity
	μl CO_2/50 min × mg nitrogen
Extract + H_2O	690
Extract + furfural	660

The conditions for measurement were those described in the section on methods except that 1×10^{-3} M furfural was added to the extracts.

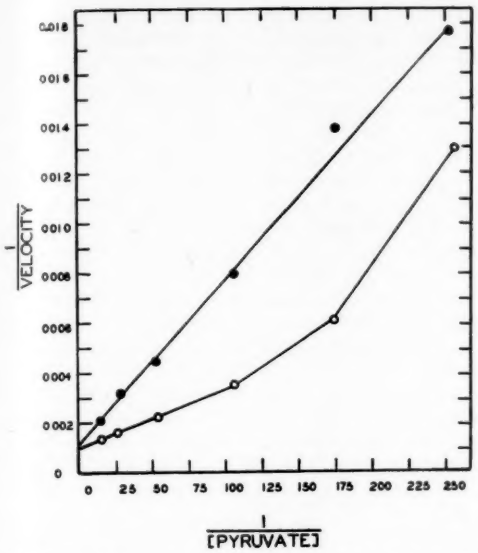


FIG. 1. Lineweaver-Burk plot of carboxylase activity in ascospores of *Neurospora tetrasperma*. Rates were calculated as μl CO_2/50 min × mg nitrogen in the extracts. All measurements were made under nitrogen, as described in the section on methods. Legend: open circles, extracts from ascospores incubated for 60 minutes after activation; solid circles, extracts of dormant spores.

carboxylase system of ascospores, their cocarboxylase content was also investigated. The extract from activated or dormant ascospores was prepared as described previously (25). Table III gives the results obtained when ascospores were incubated aerobically and anaerobically after activation. As in the case of apocarboxylase, the cocarboxylase content of activated ascospores is increased over that of dormant ones. However, anaerobically incubated ascospores, which did not germinate, even though activated, showed no such increase.

TERMINAL OXIDASES AND THE ACTIVATION PROCESS: Goddard (8) has reported that the respiration of dormant ascospores of Neurospora is much more resistant to cyanide than is that of heat-activated ones. On this basis, he suggested that a cytochrome system might arise as a result of activation, whereas such a system might be absent in dormant spores. However, he also recognized the possibility that, in the presence of a great excess of cytochrome oxidase, cyanide sensitivity might not be an accurate indicator of the presence or absence of that enzyme. Since the activity of the enzyme itself was never directly determined, it was the purpose of the next experiment to accomplish this.

Several aliquots containing 400 mg of ascospores suspended in 10 ml of distilled water were heat-activated and incubated by shaking at 28° C. After varying periods of time the ascospores were resuspended in cold 0.5 M sucrose and 0.67 M phosphate buffer, pH 7.4, and homogenized in a glass tissue homogenizer. Table IV provides the results of these experiments using dormant ascospores and those activated for varying periods of time. These results indicate that there is less than a two-fold difference between the lowest and the highest activities based on the nitrogen content of the extracts. Moreover, other experiments by the senior author have shown that this activity is associated with mitochondria-like particles, as determined by differential centrifugation experiments (unpublished results).

Tyrosinase determinations were also carried out by homogenizing ascospores in 0.05 M phosphate buffer at pH 6.5 and measuring the change in optical density at 450 mμ (26) in the presence of dopa. No activity was present in any of the extracts studied by this means or by the manometric determination of Goddard and Holden (9). Moreover, no dialyzable inhibitor of the type described by Horowitz and Shen (12) was found to be present, so that it was concluded that there was no tyrosinase present.

ANALYSIS OF INTERMEDIATES: Some insight into the nature of the metabolic block in dormant ascospores was sought through an analysis of the intermediates in cellular metabolism. For example, if the block were beyond the pyruvate stage then this compound, or related ones, might accumulate in the cell. With this in mind, chromatograms were run of extracts prepared from dormant ascospores and those

TABLE III

CARBOXYLASE CONTENT OF ASCOSPORES OF NEUROSPORA TETRASPERMA

TIME AFTER ACTIVATION (HRS)	COCARBOXYLASE CONTENT			
	μGM/MG NITROGEN		μGM/SPORE × 10^7	
	AEROBIC	ANAEROBIC	AEROBIC	ANAEROBIC
Dormant control	0.15	...	9.3	...
0	0.21	...	13.1	...
0.5	0.18	...	11.2	...
1.0	0.21	0.16	13.1	10.0
4.5	0.21	0.13	13.1	8.1

Conditions for measurement of activity as described in the text.

TABLE IV

EFFECT OF HEAT-ACTIVATION UPON THE CYTOCHROME OXIDASE CONTENT OF ASCOSPORES OF NEUROSPORA TETRASPERMA

HRS AFTER ACTIVATION	CYTOCHROME OXIDASE ACTIVITY
	μl O_2 uptake/mg nitrogen
Dormant spores	70
0	88
2	88
5	102

Experimental results presented as the average of two experiments.

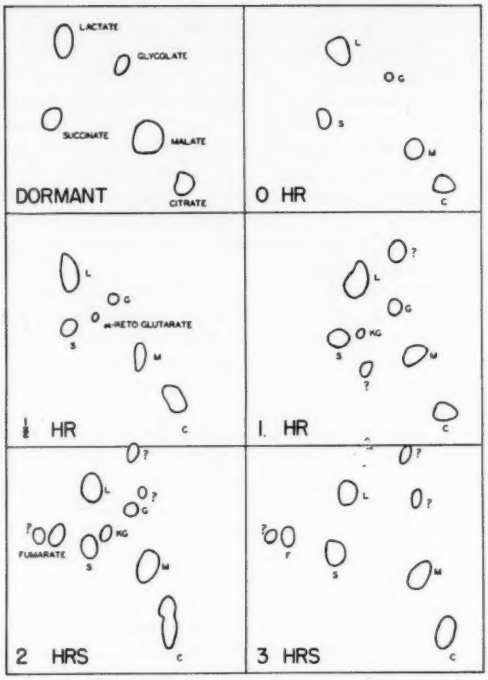


FIG. 2. Tracings of chromatograms obtained from extracts of *N. tetrasperma* ascospores that were activated and incubated for varying periods of time. The spots with question marks are those whose identification has not yet been established. The chromatograms were run in two dimensions with the alkaline solvent used first in an ascending fashion. The acid solvent was then run in a direction perpendicular to the initial direction in a descending manner. Details of solvents and preparation of extracts as described in the text. Extracts were used with no prior treatment.

activated for varying periods of time. That changes in the complement of organic acids do occur is at once apparent by the data provided in figure 2. Dormant cells contain lactate, glycolate, succinate, malate and citrate whereas after 2 hours of incubation they acquire several other acids. In addition, activated cells after 3 hours show no more glycolate. It has not yet been possible to identify the spots marked with question marks.

Due to the lability of some of the keto acids when run as the free acids or salts, they were converted to the phenylhydrazones. By this means it was found that α-keto glutarate was present in small quantities in dormant spores so its absence in extracts from such cells on the chromatogram shown in figure 2 is probably due to its decomposition.

Quantitative studies of certain of these organic acids were then undertaken, the first of which was concerned with pyruvic acid. For this purpose 500-mg samples of ascospores were ground in 5 ml of distilled water after they were activated and incubated for the indicated times. Determinations were made and the results plotted in figure 3. It should be noted that the ratio of the optical densities at 420 mμ and at 520 mμ were consistent with those reported for pyruvate by Friedemann and Haugen (6). These results clearly indicate that there is relatively little pyruvate in dormant ascospores. On the other hand, activation induces the appearance of an increased amount of this compound which is in its maximum concentration at about 120 minutes after activation. Thereafter it steadily decreases in amount. No measurable pyruvate was found in the surrounding medium at any time.

Fermentation products were now investigated since an excess of CO_2 over O_2 is known to be produced immediately upon activation. First, ethanol determinations were performed after activating and incubating ascospores for varying intervals of time. Four hundred mg aliquots of spores were incubated aerobically in 5 ml of distilled water on a shaking machine at 28° C. At the appropriate time the supernatant fluid was collected by filtration through an F-grade sintered glass filter and used for ethanol determinations with alcohol dehydrogenase. Dormant controls, incubated for two hours, were run at the same time. These results are plotted in figure 3 and show that ethanol is liberated into the medium immediately upon activation. Increasing amounts of this substance are released until about 100 minutes after activation whereupon little more is produced.

Alcohol dehydrogenase activity was then determined by incubating 250-mg aliquots of ascospores for varying periods of time after activation and preparing extracts as described in the section on methods. The extract was twice centrifuged at 12,000 × g and the precipitate discarded since it was found that the bulk of the activity was present in the supernatant. This preparation was clear amber brown in color and permitted spectrophotometric assays of the enzyme to be run as described by Racker (17). Ethyl alcohol was used as the substrate at a concentration of 0.01 M and the change in the absorption of DPN measured as described above. The results given in table V show that there are equivalent amounts of the enzyme present both before and after activation, and after one hour of incubation.

Acetaldehyde analyses showed that the ascospores

TABLE V

ALCOHOL DEHYDROGENASE ACTIVITY OF ASCOSPORES OF NEUROSPORA TETRASPERMA

HRS AFTER ACTIVATION	ALCOHOL DEHYDROGENASE ACTIVITY
	$\Delta O.D./0.1\ ml \times min$
Dormant	0.218
0	0.202
1	0.194

Details of determinations as described in text. Each figure represents the average of two determinations.

released this substance into the medium whereas the spores themselves contained only negligible amounts. For this reason, the technique described in the section on methods was evolved whereby acetaldehyde was trapped in cold bisulfite. As in the previous experiments, known volumes of ascospores were activated and incubated for varying periods of time after which the trapped acetaldehyde was determined. The results are provided in figure 3. Other experiments showed that freshly harvested spores liberated almost twice as much acetaldehyde as did those harvested nine months previously, although the shape of the curve is the same in both cases. These findings might be correlated with the differences in response to chemical activators shown by cells of different ages (23). Only small amounts of acetaldehyde are shown to be present before activation, but after this time a steady rise in its concentration occurs until about 120 minutes after activation when the curve levels off. After 300 minutes no more of this compound is liberated. Acetoin assays were carried out by the method of Westerfeld (28) but no measurable quantities were found.

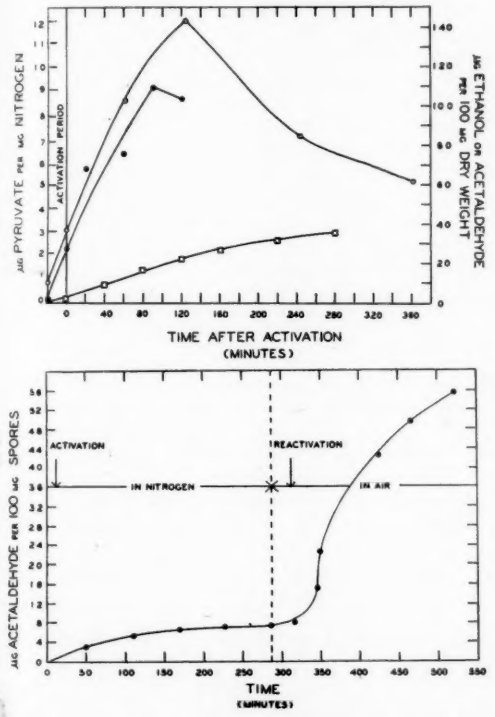


FIG. 3. Analysis of metabolic intermediates during the course of germination of ascospores of *N. tetrasperma*. The pyruvate content in spore extracts was determined while the ethanol and acetaldehyde analyses were of the suspending medium. Legend: open circles, pyruvate; solid circles, ethanol; squares, acetaldehyde.

FIG. 4. Effect of anaerobiosis and subsequent reactivation and return to air upon acetaldehyde production by ascospores of *N. tetrasperma*. The analyses were made of the suspending medium.

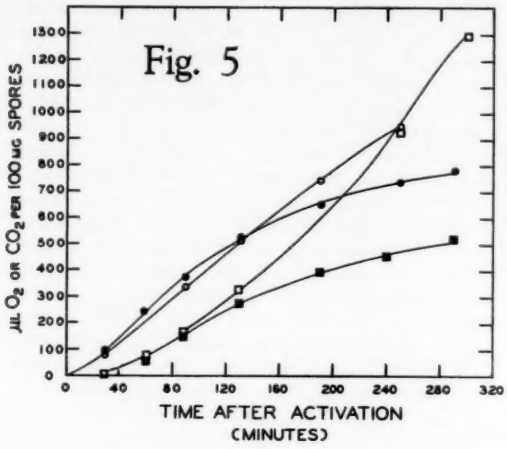
Goddard (8) has shown that anaerobic incubation of activated ascospores results in their reversible "deactivation." Therefore, a stream of washed nitrogen was passed through a suspension of activated ascospores for 290 minutes. An air stream was then reintroduced and the spores were again heat-activated after this time. The amount of acetaldehyde produced under these conditions is shown in figure 4. Under anaerobic conditions, acetaldehyde production is greatly reduced but reactivation and incubation in air causes the rapid release of this compound. The rate of release falls markedly after 60 minutes and the total acetaldehyde produced exceeds that of ascospores incubated in air from the start.

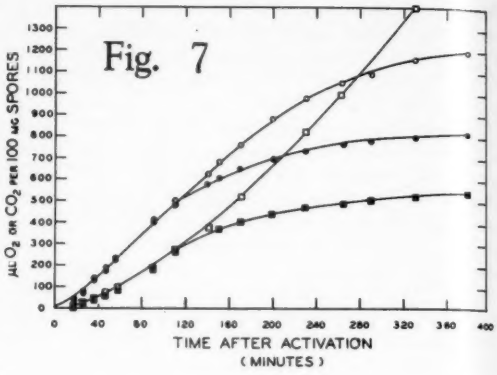
EFFECT OF METABOLIC POISONS: Previous work has shown that very low concentrations of cysteine (unpublished data of the senior author) were toxic to ascospore germination. Therefore, this substance was added to ascospores in a final concentration of 1×10^{-3} M and their respiration and acetaldehyde production measured. As can be seen in figure 5, cysteine markedly reduced both oxygen consumption and carbon dioxide evolution within 90 to 100 minutes after activation. In addition, germination was completely suppressed. Acetaldehyde production was, however, immediately increased as a result of such treatment, as figure 6 shows. Moreover, the compound is produced over a longer period of time in cysteine-treated cells so that a possible locus of the poisoning effect might lie in those steps devoted to the utilization of pyruvate. That such is a reasonable possibility is suggested by the data of Rubinstein and Denstedt (18) who found that cysteine was toxic to the condensing enzyme system. This system is concerned with the formation of citrate by the condensation of oxaloacetate and a 2-carbon fragment, presumably acetyl coenzyme A.

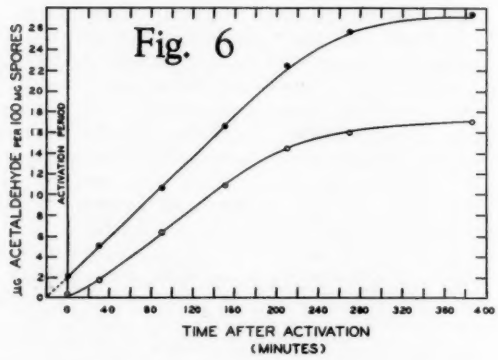
To test this hypothesis, 1×10^{-2} M fluoroacetate was added to ascospores before activation and their respiration and acetaldehyde production was studied as before. This substance is more or less specific for the steps immediately following the condensing enzyme (16) so that, according to the above, one might expect to observe the accumulation of acetaldehyde as in the case of cysteine-treated cells. That this is indeed the case is seen in figures 7 and 8 wherein the respiratory processes are shown to be inhibited in the same way as described above. Moreover, acetaldehyde accumulates in quantities greater than in the controls with the difference that its production does not drop off markedly, even after 480 minutes. Finally, germination is also entirely suppressed.

DISCUSSION

These results bear upon both the mechanism of activation and upon the metabolic transition from the germinating to the vegetative condition. As for the first of these, the near-equivalence of both apo-







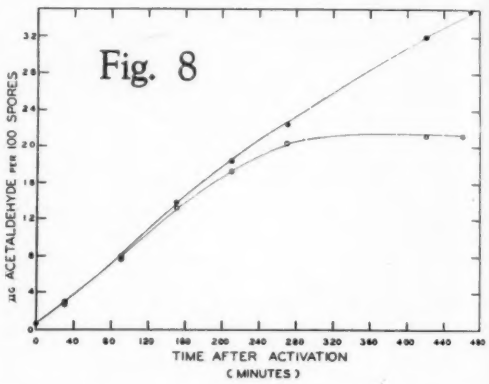


FIG. 5. Effect of cysteine (1×10^{-3} M) upon the respiration of Neurospora ascospores. Legend: open squares, O_2 uptake of controls; open circles, CO_2 output of controls; closed squares, O_2 uptake of treated cells; solid circles, CO_2 output of treated cells.

FIG. 6. Acetaldehyde production by Neurospora ascospores after treatment with 1×10^{-3} M cysteine. Legend: open circles, control; solid circles, treated cells. Analyses were of the suspending medium.

FIG. 7. Effect of fluoroacetate (1×10^{-2} M) upon the respiration of Neurospora ascospores. Legend: open squares, O_2 uptake of controls; open circles, CO_2 output of controls; closed squares, O_2 uptake of treated cells; solid circles, CO_2 output of treated cells.

FIG. 8. Acetaldehyde production by Neurospora ascospores after treatment with 1×10^{-2} M fluoroacetate. Legend: open circles, control; solid circles, treated cells. Analyses were of the suspending medium.

carboxylase and cocarboxylase in dormant and activated ascospores argues against their direct participation in the activation of ascospores.

This is corroborated by the data of Goddard (unpublished results) who obtained similar results. Certainly, even a 2-fold increase cannot explain the 20-fold respiratory increases which are noted upon activation. Moreover, were this enzyme alone to be the metabolic block in dormant cells then it might be expected that the product of its activity, acetaldehyde, would activate such spores. This, however, is not the case even though related compounds like pyruvate, acetate, and furfural penetrate. Goddard's data showing the reversal of fluoride poisoning of the activation process with pyruvate could be explained by assuming that fluoride poisons a step which is later than the actual activation step. Therefore, on this basis, and assuming an active glycolytic system, a step anterior to enolase could be the one involved most directly in the activation of Neurospora ascospores. Alternatively, another pathway could be involved.

The change in the slope of the Lineweaver-Burk plot of carboxylase activity of activated ascospores suggests that systems competing for pyruvate as a substrate exist in such cells. It is interesting to note that the K_m obtained by Strauss (22) for carboxylase extracted from the vegetative mycelium of mu-

tants of *Neurospora crassa* was about 2×10^{-3} as compared with 4.2×10^{-2} and 0.9×10^{-2} for dormant and activated ascospores respectively on *N. tetrasperma*. It is possible, therefore, that the activated ascospores possess a pyruvic carboxylase that more closely resembles that of the vegetative cells of Neurospora.

That terminal oxidases are not directly involved in the breaking of dormancy is suggested by the data obtained with cytochrome oxidase. However, it must be admitted that these results, taken by themselves, are not sufficient evidence for the participation of cytochrome oxidase in the respiratory system of the ascospores. It may be that the enzyme is present but is not functioning in the dormant ascospore so that the cells in this condition are not sensitive to cyanide (8). Therefore, the transition from the dormant to the germinating condition may involve the induction of a cytochrome-dependent system.

The metabolic steps involved in the germination process, as contrasted with activation itself, are becoming clearer. Figures 5 and 7, and previous data (7, 23) show the production of an excess of carbon dioxide compared to oxygen uptake. The disparity between these variables is most marked during the first sixty minutes after activation, but thereafter the rate of oxygen uptake soon exceeds that of carbon dioxide evolution. Paralleling the high rate of fermentation at the start of germination is the accumulation of pyruvate in the cell, and the liberation of ethanol and acetaldehyde into the medium (fig 3). As in the previous case, the rate of accumulation and release of these intermediates soon falls and at about 120 minutes after activation these entirely cease. In the case of acetaldehyde production it should be noted that both the rate and total amount released are dependent upon the age of the ascospores. This fact may have some bearing upon the loss in the ability of "aged" ascospores to respond to furfural, although the connection between these observations is still not clear.

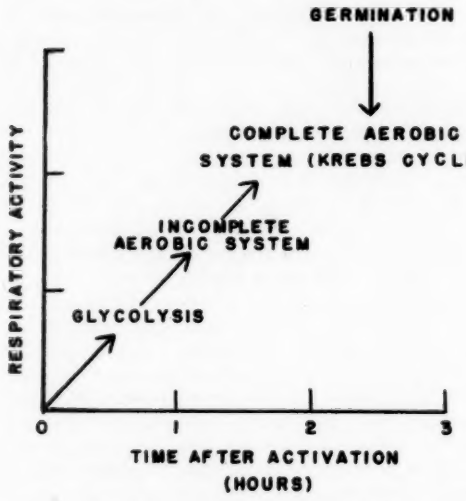
Up to this point it is suggested that ascospores, immediately upon activation, glycolyze at a rapid rate as compared with dormant cells. However, this rapid fermentative rate is not maintained under aerobic conditions, as was discussed previously, nor is it maintained anaerobically. This is established by the data of Goddard (8) who showed that carbon dioxide output, as well as germination, was restricted in an atmosphere of nitrogen. In addition, the data in figure 4 confirm this observation by showing that acetaldehyde release is greatly restricted as well. On the other hand, reactivation of such ascospores and their return to air results in the rapid release of acetaldehyde which is accumulated in greater amounts than in the case of controls maintained in air. It is suggested that anaerobic incubation of activated ascospores induces the accumulation of a product or products which is converted into acetaldehyde upon reactivation and incubation in air.

Concomitant with the decrease in the accumulation of ethanol and acetaldehyde, products of oxidative metabolism begin to appear for the first time or in larger amounts. Such substances include fumarate and alpha ketoglutarate. In fact, the only intermediates in the Krebs cycle that have not yet been identified chromatographically are oxaloacetate and cis-aconitate and the extreme lability of the former may render it difficult to analyze by this means. The absence of oxaloacetate has also been noted recently in conidia of *Neurospora sitophila* (15). Lactate, however, was not found to be present in these conidia whereas its presence in dormant ascospores has been verified both chromatographically and colorimetrically (unpublished results). Another difference between ascospores and conidia lies in the fact that the former accumulate glycolate and the latter glyoxylate. It is, of course, possible that both compounds exist in the ascospores since there were several unresolved spots present on the chromatograms. Although the significance of these observations is as yet uncertain, it may be that the glycolic acid oxidase system is functional at some stage of ascospore development despite the inability of Cheng (4) to find it in dormant ascospores.

The poisons used in these experiments act similarly with respect to their effect upon gas exchange and germination: in both cases, marked inhibition results, and at approximately equivalent times after their introduction to the spores (cf figs 5 and 7). However, some differences in their effect upon acetaldehyde release are aparent from the data provided in figures 6 and 8. For example, cysteine-treatment results in the immediate release of larger amounts of acetaldehyde than the control for at least three hours after activation, after which both rates markedly decline. In contrast, no effect on acetaldehyde release is manifested by fluoroacetate until after almost three hours of incubation. In this case the poisoned cells continue to release acetaldehyde while the control rate declines as described previously. The common effect of both treatments is the release of larger amounts of acetaldehyde, a result which would be in keeping with the notions of Peters (16) and Rubinstein and Denstedt (18) that these compounds poison reactions associated with the condensation of pyruvate and acetyl coenzyme-A as well as with the utilization of citrate in the Krebs cycle. Since both poisons prevent germination, it is suggested that these metabolic reactions must be developed before germination can be completed. It must be conceded that the results with inhibitors are open to the criticism that their delayed toxicity may be due merely to their failure to penetrate. However, the change in the RQ and in the nature of the organic acids accumulated are independent evidence in favor of a change in the type of metabolism.

It is proposed, therefore, that immediately upon activation the cell rapidly glycolyzes and produces ethanol and acetaldehyde. Concomitantly, the cell develops oxidative means for the utilization of pyruvate, namely the condensing enzyme system and per-

haps the Krebs cycle, whereupon the release of ethanol and acetaldehyde gradually is restricted. Finally, the transition to the aerobic type of metabolism occurs, with the result that the vegetative stage of the organism is initiated with the complement of respiratory enzymes present as partially delineated by Strauss (21) and Cheng (4). It is worth noting that Strauss has provided evidence for the presence of two routes of pyruvate oxidation in the vegetative mycelium of this mold. In outline form this sequence of events would appear as follows:



Although no visible sign of germination can be noted until after the aerobic type of metabolism appears, it is likely that there is, at least, a functional alteration of the cell surface before the germ tube is protruded. This possibility is supported by previous data showing that marked permeability changes occur at this time resulting in the release of Ca^{++} and K^{+} from the ascospores in the presence of versene (24).

It is recognized that the unequivocal proof of this proposed scheme must come through the isolation of the enzymes involved, but it seems definitely established that the activation step is only the beginning of a sequence of metabolic adjustments which culminate in the development of the vegetative cell.

Summary

1. The metabolic changes induced by the breaking of dormancy in Neurospora ascospores were investigated by means of enzymatic, chromatographic and poisoning techniques.

2. The near-equivalence of both the apoenzyme and coenzyme in dormant and activated ascospores suggests that the lack of pyruvic carboxylase is probably not the reason for the metabolic block preventing their germination.

3. Ethanol dehydrogenase and cytochrome oxidase activities were also shown not to change very markedly as a result of activation.

4. Paper chromatographic analysis of dormant ascospore extracts disclosed the presence of lactate, glycolate, pyruvate, citrate and malate. On the other hand, extracts from activated cells showed fumarate, increased amounts of alpha ketoglutarate, and several unknown spots in addition to the acids found in dormant cells.

5. Acetaldehyde and ethanol, although present in minimal amounts in dormant ascospores, are produced in relatively large amounts immediately after activation. However, after 2 hours of incubation, activated cells no longer produce these fermentation products.

6. Fluoroacetate and cysteine poison germination and respiration but only after 2 hours of incubation subsequent to activation.

7. These data are taken to indicate that activation induces the origin of a glycolytic system which may work through the Krebs cycle. Furthermore, germination requires the induction of an oxidative type of metabolism which is different from that found in dormant ascospores.

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DEHYDROGENASE ACTIVITY OF HYDROXYMALONATE AND RELATED ACIDS IN HIGHER PLANTS[1,2]

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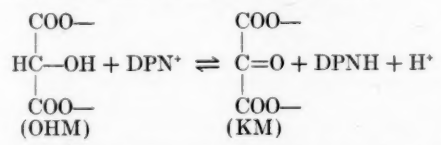
Plant extracts have been found to catalyze the following reversible reaction dependent upon diphosphopyridine nucleotide (DPN), in which hydroxymalonate (OHM) is oxidized to ketomalonate (KM):



The above compounds are also known as tartronic and mesoxalic acids respectively.

KM has been reported to be present in the leaves of alfalfa, *Medicago sativa* (11), and both KM and OHM have been identified in sugar syrups made from plants (21). OHM has also been isolated from cultures of *Acetobacter acetosum* grown on glucose medium, but this might be due to a non-enzymatic decomposition of 2-keto-D-gluconic acid during the heat treatment with alkali (17).

Using methylene blue as an indicator of dehydrogenase activity, Quastel and Woolridge (22) reported activity with OHM by whole cells of *Escherichia coli*. OHM was found to be an inhibitor of lactic dehydrogenase in *E. coli* (22) and in guinea-pig brain slices (19), and also a competitive inhibitor of malic dehydrogenase of pig-heart tissue (16) and of a non-DPN dependent malic oxidation in pigeon-liver extracts (25). The latter pigeon-liver extracts did not oxidize OHM either with or without added DPN. Recently, OHM has been considered as a possible coenzyme of oxalosuccinic carboxylase (31).

The present study was undertaken because of the posible interrelationships of OHM and KM with two other substrates associated with a DPN-dependent dehydrogenase activity in plants, i.e., diketosuccinate and dihydroxyfumarate (26).

Materials and Methods

The wheat germ enzyme was prepared from wheat germ S-50 (kindly supplied by General Mills) by extracting an acetone powder in 8 times its weight of M/200 phosphate buffer (pH 7.4). Subsequent treatment with $MnCl_2$ and solid ammonium sulfate was similar to that reported for phosphogluconic dyhydrogenase (2), except that the fraction used was that obtained between 200 to 400 gm ammonium sulfate per liter of enzyme solution. At the last step, the ammonium sulfate precipitate was taken up in approximately 1/5 the original volume and the resulting fluid

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contained about 100 mg of protein per ml (29). Dialyzed extracts of acetone powders (27) were used in the distribution study.

KM was used from 3 sources, with no essential chemical or enzymatic difference between these purified samples. Most of the experiments were performed with the sodium salt of KM purchased from Aldrich Chemical Company and recrystallized from a saturated aqueous solution of the sodium salt by the addition of alcohol. The 2,4-dinitrophenylhydrazone derivative had a melting point of 202 to 204° C (24), and an R_f value of 0.40 to 0.45 when chromatographed in the butanol phase of a butanol–alcohol–water (50–10–40 parts by volume) solvent, using ascending chromatography. Only one yellow spot was obtained which turned reddish-brown upon spraying with alcoholic KOH. The phenylhydrazine derivative had a melting point of 163 to 165° C (24), and a R_f value of 0.54. A second sample was obtained as the barium salt from alloxan, using the method of Deichsel (9). A third sample prepared from ethylmalonate (8) was kindly supplied by Dr. I. Zelitch. (He reported that he had obtained the 2,4-dinitrophenylhydrazone with the correct melting point in better than a 92 % yield by weight.)

KM was identified chromatographically as a pH spot after spraying with brom-cresol green (3) and as a brown-black spot after spraying with alkaline $AgNO_3$ (3) with R_f values of 0.2 in the mesityl oxide phase of a mesityl oxide–formic acid–water solvent (6). It is possible to spray first with the pH indicator, followed with the $AgNO_3$ spray to observe the superimposed spots. The 2,4-dinitrophenylhydrazone was identified chromatographically using the method of Cavallini (7), and analyzed quantitatively using the total hydrazone method (15) with an incubation period of 1 hour (the color increases with even longer incubation periods) with a broad peak between 455 to 460 mμ. KM was also estimated by the α-methylindole colorimetric method of Dische (10). A yellow color is formed in Feigl's naphthoresorcinol test (12) modified for colorimetric analysis as for glyceric acid (28). Decarboxylation of the sodium salt with an insoluble yeast decarboxylase (4, 5) produced glyoxylate, which was identified chromatographically as a pH spot and as a red color in the phenylhydrazine–potassium ferricyanide test (5, 14).

Hydroxymalonate was prepared from diketosuccinate (prepared from tartaric acid) according to Fenton (13) with a melting point of 158 to 160° C (24), or was purchased from the Aldrich Chemical Company. It was necessary to purify the commercial samples by Norite treatment followed by recrystallization from concentrated solution before crystals were obtained with the above melting point. Chemical analysis of one of these samples gave 29.5 % C, 3.51 % H; expected analysis for anyhydrous OHM; 30 % C, 3.33 % H. (These analyses were made through the kindness of Dr. B. Vennesland.) Recrystallization of the Norite treated acid as the potassium salt by the addition of alcohol was a more convenient method of purification. Earlier commercial samples sold in the hydrate form were contaminated with an enzymatically active compound similar to or identical with tartaric acid, while recent anhydrous samples have been free of this compound. As both DL- and meso-tartaric acids are also enzymatically active, it was necessary to check the sample of OHM for contaminating tartaric acid.

The following tests were useful in differentiating between these two acids. DL- or L(+)-tartaric acid forms an insoluble potassium acid salt without the addition of alcohol. About a 50 % alcoholic solution is necessary to precipitate the potassium acid salt of OHM. (Alcohol is also necessary in the case of meso-tartaric acid.) OHM reduces ammonium molybdate solution to blue upon heating while tartaric acid does not react. The blue color can be observed chromatographically by spraying with the molybdate spray reagent as prepared by Bandurski and Axelrod for phosphate compounds (1), and heating the paper for 5 minutes at 85° C. This reducing method can be adapted for colorimetric estimation by the addition of 0.1 ml of the molybdate solution to the sample (0.1 micromole of OHM) in 1 ml of water. After heating in a hot water bath at 85° C for 10 minutes, the blue color is measured colorimetrically at 660 mμ. KM can be identified as the main product after heating an acid solution of OHM in the presence of an equimolar solution of $CuSO_4$. A yellow color with a broad absorption peak at 430 mμ is formed in the Feigl naphthoresorcinol test at a concentration of 5 micromoles of OHM per ml. This is in contrast to the greenish color reported by Feigl (12). Tartaric acid gives a bright green color with the highest absorption peak at about 680 mμ and a lower one at 430 mμ at a concentration of 0.5 micromoles per ml. Furthermore, the two acids can be separated chromatographically, using a mesityl oxide–formic acid–water solvent combination (6) with the following R_f values: OHM 0.38, tartaric 0.2, KM 0.2, malic 0.42, glyoxylic, 0.22, diketosuccinic 0.39 (presumably decomposing to OHM). An ether–formic acid–water solvent (100–40–20 parts by volume) likewise is useful in differentiating between these two compounds: OHM 0.65, tartaric 0.49, KM 0.39, malic 0.68.

Even the purest preparations (correct melting point) show very faint pH spots near the origin (R_f 0.03) when large amounts (greater than 0.5 micromoles) are chromatographed. This faint spot does not react with the molybdate or $AgNO_3$ spray. Old samples, frozen and thawed several times, indicate an increase in this unidentified spot, and KM samples showed similar slow-moving spots in old solutions.

DPN of 90 % purity was purchased from Krishell's Chemical Company. DPNH of 90 % purity as a TRIS (tris(hydroxymethyl)aminomethane) salt was prepared by Dr. F. Loewus. DL- and meso-tartaric acids were purified as K acid salts from samples purchased from Aldrich Chemical Co. L(+)-tartaric acid (Merck tartaric acid, reagent grade) was similarly purified. DL-malic acid from Krishell Chemical Co.

was recrystallized from ethyl acetate with petroleum ether.

Experimental Results

Direct Spectrophotometric Observation of DPNH Oxidation and DPN Reduction: Typical data showing reduction of DPN^+ spectrophotometrically in the presence of added OHM are shown in figure 1, using a wheat germ enzyme preparation. DL- and meso-tartaric acids give similar DPN reduction rates and final equilibrium values at approximately one-half the substrate concentration, assuming that the activity with DL-tartaric acid is due to the D(−)-form only. The L(+)-isomer alone is inactive, just as it has been reported for liver mitochondria preparations (18). With both OHM and tartaric acids, the final DPN reduction was similar aerobically and anaerobically.

Equilibrium and Michaelis constants were calculated from the above data. As these values were obtained with a crude enzyme with a high blank reaction that was subtracted from all of the data, these constants should only be considered as rough estimates. The K value for the reaction $(KM)(DPNH)(H^+)/(OHM)(DPN^+)$ was equal to 1×10^{-14} M, while K_m for OHM at pH 9 was equal to 3.8×10^{-1} M.

In the presence of added KM, DPNH is rapidly oxidized. This activity could not be due to the decarboxylation of KM to glyoxylate and the subsequent oxidation of DPNH by glyoxylic reductase (32) because the wheat germ enzyme preparation which is active with KM is relatively inactive with added glyoxylate. Furthermore, the subsequent addition of KM to the glyoxylate vessel causes a rapid oxidation of the DPNH at a rate comparable to that of the cuvette with KM alone. Similar results were obtained with a pea shoot enzyme preparation, although the initial glyoxylate activity was higher. No significant difference was observed when KM was added under anaerobic conditions.

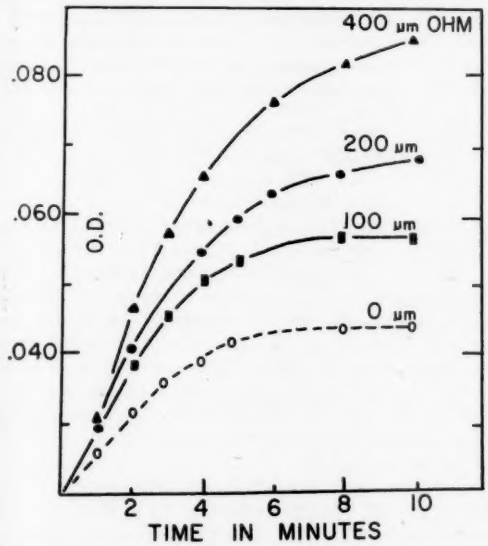

Fig. 1. Reduction of DPN^+ in the presence of wheat germ enzymes by OHM or tartaric acids. The second curve from the top (solid circles) represents a family of curves for the reduction of DPN^+ in the presence of 200 micromoles OHM, 200 micromoles DL-tartaric, or 100 micromoles of D(−)-tartaric acid. The other curves represent data for OHM only. These experiments were made in 0.08 M TRIS buffer at pH 9.0 in a 3-ml volume containing 0.4 mg DPN^+, 0 to 400 micromoles of OHM or tartaric acid, and 20 mg of wheat germ enzyme. Optical density (OD) measurements were made at 340 mμ.

Table I

KM Disappearance and OHM Formation by OHM Dehydrogenase Activity Coupled with Alcohol Dehydrogenase

Time	KM disappearance	OHM formed
min	*micromoles*	*micromoles*
0	0	0
60	34.6	34.0
180	40.8	43.2

The experimental vessels contained 0.2 ml of 0.5 M TRIS buffer at pH 7.4, 0.4 mg DPN^+, 75 micromoles of KM, 0.1 ml 95% ethanol, 51 mg of wheat germ enzyme in a total volume of 3 ml.

Experiments with Coupled Systems: The addition of OHM to wheat germ extracts can be coupled with the reduction of 2,6-dichlorophenolindophenol presumably via a diaphorase also present in the extract. Comparable amounts of D(−)-tartaric acid added as the DL- form were also able to reduce the dye.

The reduction of KM to OHM could be coupled with the oxidation of ethanol to acetaldehyde in the presence of DPN. Table I shows typical data from such an experiment in which the disappearance of KM was compared with the appearance of OHM. At the end of the indicated incubation periods, the vessels were held in a hot water bath for 10 minutes in order to drive off the acetaldehyde, and the cooled extract was acidified with 0.1 ml of concentrated sulfuric acid and was centrifuged. Then, 0.05-ml aliquots were analyzed for KM by the α-methylindole test or total hydrazone method, and 0.1-ml aliquots for the molybdate reducing activity of OHM. Values were standardized against standards added to non-incubated vessels with all of the components of the experimental vessels. In a number of different experiments using wheat germ or parsley leaf enzymes, about 50% of the added KM disappeared.

Identification of OHM Starting with KM: Starting with KM as the initial substrate, the enzymatic production of OHM was identified chromatographically in both coupled and uncoupled systems. While OHM can be identified in the crude incubation system after chromatography by using the molybdate spray method, further purification is necessary in order to identify it as the free acid because of interference with other acids of the mixture.

A typical large scale experiment was performed as follows: an incubation mixture in a 4-ml volume was prepared containing the following components: 2.0 ml of an 0.5 M phosphate buffer (pH 7.4), 300 micromoles of DPNH as the TRIS salt, 300 micromoles of the sodium salt of KM, and 13 mg of a purified protein from parsley leaves. The reaction was followed spectrophotometrically at 340 mμ until the reaction was completed. The reaction mixture was acidified by the addition of 0.3 ml of 18 N H_2SO_4, followed by continuous ether extraction for 60 hours from this aqueous medium. The enzymatic product co-chromatographed with OHM in 5 different solvent combinations. The pH spot and the blue molybdate spot were superimposable. Controls without DPNH and without KM showed no such spots.

Identification was likewise made in a coupled system, using both ether extraction and anion column purification in order to isolate the acid. An incubation mixture similar to that described in table I was analyzed by Dische's α-methylindole test, indicating that 50 % of the KM had disappeared. The remainder of the sample was air dried on strips of filter paper (30 × 4 cm). These strips were folded into a cylinder and inserted into a Soxhlet extractor in place of the thimble and were extracted with ether for 8 hours. Further separation by anion column chromatography was necessary in order to separate the remaining KM from the OHM. The ether extract was taken up in water after evaporation to dryness and put on a Dowex-1 (or IRA-400) column (about 1 × 10 cm) in the carbonate form (made with 5 % Na_2CO_3). After washing with water, the column was eluted with 100 ml of 2.5 % $(NH_4)_2CO_3$. (The KM remains on the column and can be eluted with 5 % $(NH_4)_2CO_3$.) The 2.5 % eluate was evaporated almost to dryness on a hot plate at low temperature. After batchwise decationization with Dowex 50 (H^+ form), aliquots were chromatographed. Again the molybdate-reducing spots superimposed the pH spots, and co-chromatographed with known OHM in several solvent combinations. Control experiments were negative.

Effect of OHM on Malic Dehydrogenase Activity: Since OHM has been reported to be a competitive inhibitor of lactic and malic dehydrogenases of animal tisues (16, 19, 22, 25), the inhibition of wheat germ malic dehydrogenase was checked. At an enzyme concentration so dilute that no OHM activity can be demonstrated, the addition of OHM to malic acid showed a typical competitive inhibition as indicated in figure 2. Rates were calculated from the first 5 minutes of activity after the DPN was added. These data are graphed as a double reciprocal plot of velocity vs substrate concentration according to Lineweaver and Burk (20). Blank values of increase of absorption at 340 mμ without added substrate were negligible.

Calculations of the Michaelis and Menten constant using these data give a value for K_m of about 1×10^{-2} M for the DL- form of malic acid, or presumably one-half of this value for the L- form. Other calculations

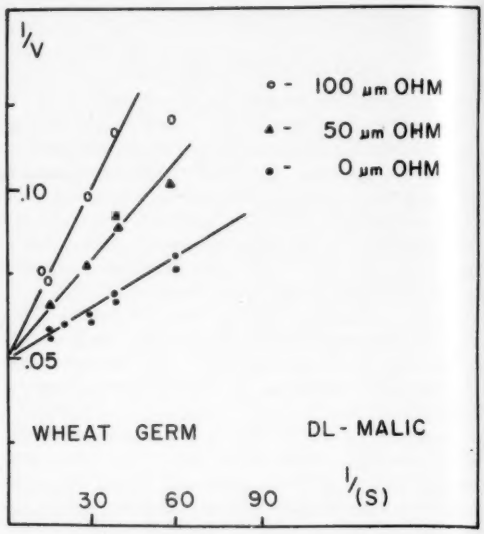

Fig. 2. The competitive inhibition of malic dehydrogenase activity in wheat germ by OHM. The incubation mixtures contained in a 3-ml volume 0.5 ml TRIS buffer (pH 9), 0.05 ml freshly prepared 0.1 M KCN, 50 to 200 micromoles DL-malic acid, 50 or 100 micromoles OHM, 10 μgm wheat germ protein, and 0.4 mg DPN^+ added in that order. V = velocity, expressed as the change in optical density per minute at 340 mμ; S = molar substrate concentration of DL-malic acid.

for K_m for the L- form are those reported for wheat seeds with a K_m equal to 4×10^{-2} M (30), and for animal tissues with a value close to 1×10^{-2} M (16). The value of K_i for OHM (20) was equal to 1×10^{-2} M, indicating that OHM is less active an inhibitor in the wheat germ system than in pigeon liver (25).

Distribution of OHM Activity in Plants: The dehydrogenase activity capable of converting KM to OHM is probably widely distributed in plants, because it is present in all parts of a plant during various developmental stages. The activities with oxalacetate and diketosuccinate have a similar distribution within a plant (table II), while the activities with glyoxylate or hydroxypyruvate are found predominantly in leaves (27).

Table II shows enzymatic data for parts of 5 different plants. Since oxalacetic and diketosuccinic acids are unstable, they were added as small amounts of solid. The oxidation of DPNH without added substrate was subtracted from the total change in optical density with added substrate. The enzyme was diluted so that the change in optical density was not higher than 0.020 per minute. The reaction was followed for 10 minutes, and the activity expressed as the change in optical density × $minutes^{-1}$ × $mg\ protein^{-1}$ × 100. All enzyme preparations were made from acetone powders. Similar preparations give values of about 30 for leaf enzyme activity with hydroxypyruvate (28). Wheat germ and pea root preparations

TABLE II

DISTRIBUTION OF DEHYDROGENASE ACTIVITIES IN HIGHER PLANTS

PREPARATION	KETO-MALONATE	DIKETO-SUCCINATE	OXAL-ACETATE
Pea shoot	8.7	10.2	310.0
Pea root	21.7	8.1	694.0
Pea seeds	2.8	0.8	120.0
Parsley shoots	1.8	1.5	100.0
Watercress shoots ..	6.5	2.4	337.0
Carrot root	0.6	0.2	37.4
Wheat root	3.0	2.6	105.0
Wheat germ	20.9	10.9	730.0

The data were obtained spectrophotometrically at 340 mμ in a 3-ml volume containing 0.2 ml 0.05 M phosphate buffer (pH 7.4), 200 micrograms DPNH, 10 micromoles KM or approximately 10 micromoles of diketosuccinic or oxalacetic acids as solids. Activity is expressed as the change in optical density $\times$ min^{-1} $\times$ mg $protein^{-1}$ $\times$ 100.

were among the best sources for all 3 enzymatic reactions.

PURIFICATION OF PARSLEY LEAF ENZYME: A partial purification of the parsley leaf preparation was undertaken to determine whether the activities for oxalacetate and KM could be separated. Although possessing a lower activity with KM than wheat germ, parsley was used because the initial purification steps are easier. Table III shows enzymatic data for some of the fractions in the purification process. The procedure was devised for the best specific activity for KM, but certain of the fractions were tested for the other activities as indicated. The first few fractions were also tested for activity with hydroxypyruvate. This activity was rapidly eliminated by the pH 5 treatment and subsequent steps.

An acetone powder made from a commercial source of parsley shoots was extracted 1 hour in an 0.001 M phosphate buffer, pH 7.4, and strained through cheesecloth and centrifuged at low speed. To this supernatant (H) were added 400 gm of solid ammonium sulfate per liter of supernatant. The centrifuged precipitate was taken up in a minimum volume of buffer and dialyzed against 0.001 M phosphate buffer (P_1). Then, 200 gm $(NH_4)_2SO_4$ per liter were added to the solution of P_1. The new precipitate, P_2, was subsequently discarded. More $(NH_4)_2SO_4$ was added to the above solution to make a total of 400 gm per liter of solution. The precipitate, P_3, represents the ammonium sulfate precipitate obtained between 200 to 400 gm per liter. The supernatant was discarded. Fraction P_3 was taken up in distilled water and the pH lowered to 5.0 with 10 % acetic acid. The residue was discarded. The supernatant, S_1, contained approximately 75 % of the activity of the extract H, and was re-fractionated with ammonium sulphate. The first precipitate between 0 to 250 gm per liter was discarded. The second precipitate (P_4) between 250 to 300 gm per liter contained approximately 35 % of the activity of H, and the third precipitate (P_5) from 350 to 400 gm per liter about 26 % of the original activity. These two fractions represented a 30–50-fold purification. Subsequent treatment with calcium phosphate gel increased the total purification about 80-fold, but in fractions representing only about 1 % of the original activity of H. About 70 mg of $Ca_3(PO_4)_2$ gel were added to 40 mg of the protein in fraction P_4. The supernatant S_2 had one of the highest specific activities (146) but represented only 1 % of the original total activity. The enzyme adsorbed on the gel was eluted with 3 successive 5 ml portions of 0.1 M phosphate buffer (G_1, G_2 and G_3) containing 12 %, 7 %, and 1 % of the activity of H, respectively.

Although the three enzymatic activities with oxalacetate, diketosuccinate and KM follow the same general pattern during purification, some differences were found. The highest purification was about 80-fold for KM, 48-fold for oxalacetate, and 28-fold for diketosuccinate.

TABLE III

SUMMARY OF DEHYDROGENASE ACTIVITIES DURING THE PURIFICATION OF A PARSLEY LEAF PREPARATION

PREPARATIVE STAGE	KETO-MALONATE	DIKETO-SUCCINATE	OXAL-ACETATE
H	1.8	1.5	100
P_1	11.1	...	....
P_2	1.9	...	....
P_3	20.5	...	....
S_1	38.0	5.0	940
P_4	52.5	6.8	1669
P_5	95.8	...	....
S_2	146.0	42.4	4781
G_1	97.0	19.0	1735
G_2	91.0	28.0	1757
G_3	140.0	25.0	2765

Activity is expressed as in table II, and the purification stages are described in the text.

DISCUSSION

Is the dehydrogenase activity reported here for OHM of any physiological importance? Although OHM has been identified in biological material (11, 21, 23), the recent finding of OHM as a non-enzymatic product of a gluconic acid derivative during the extraction of the acid (17) indicates the possibility of a non-enzymatic origin of this compound in extracts of biological material. This problem is still to be reinvestigated.

A second problem is whether this activity with OHM is due to a separate enzyme or to the activity of a relatively non-specific dehydrogenase. Malic dehydrogenase has a similar distribution pattern in plants, but the ratio of the activity with oxalacetate and KM does vary somewhat during purification. Furthermore, the competitive inhibition by OHM of malic dehydrogenase activity at a level of enzyme concentration too weak to give any activity with OHM alone, would argue against the activity being due to the malic dehydrogenase. Complete separation of the enzymatic activities will be necessary to prove this point.

If one assumes a physiological function for OHM and KM, their relationships with closely related compounds are of interest. The following conversions might be involved: D-tartrate $\xrightarrow{-2H}$ dihydroxyfumarate $\xrightarrow{-2H}$ diketosuccinate $\xrightarrow{-CO_2}$ OHM $\xrightarrow{-2H}$ KM. There is evidence of enzymatic activity in plant extracts for all but the decarboxylation step. Although leaf extracts possess an active hydroxypyruvic reductase activity (28), no evidence has been obtained in these leaf preparations of any hydroxypyruvate arising from dihydroxyfumarate as reported by Kun and Hernandez (18).

Summary

A dehydrogenase activity capable of a reversible conversion of hydroxymalonate to ketomalonate in the presence of diphosphopyridine nucleotide has been demonstrated in a variety of plant extracts. The distribution of this activity in plants is similar to that for diketosuccinic reductase and malic dehydrogenase. An 80-fold purification of the enzyme was made. Although oxalacetate and diketosuccinate activities were still present, changes in relative activity occurred, indicating that different enzymes are involved. Furthermore, hydroxymalonate is a competitive inhibitor of malic dehydrogenase activity at enzyme concentration levels too weak to give any activity with hydroxymalonate alone. Although both ketomalonic (mesoxalic) and hydroxymalonic (tartronic) acids have been demonstrated by others in plant extracts, the physiological role, if any, of this activity is still to be demonstrated.

The author is indebted to Dr. B. Vennesland, Biochemistry Department, University of Chicago, under whom the earlier work was done, and to Dr. A. Magaldi who performed some of these earlier experiments.

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THE REACTIONS OF THE PHOTOINDUCTIVE DARK PERIOD[1,2]

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Since the demonstration by Hamner and Bonner (7) of the importance of the dark period for photoperiodic induction in the short-day plant Xanthium, there have been many attempts to elucidate the reactions which take place within the plant during this period (2, 11, 13, 18). The dark period reactions appear to be concerned directly with the act of induction—the persistent change of the plant from the vegetative to the flowering condition. In Xanthium the flowering condition persists after a single completed act of induction even though the photoperiod during floral development is too long for induction to occur in vegetative plants. The condition of the plant after induction has taken place will be termed the induced state to distinguish it from the act of induction.

The induced state in Xanthium is a quantitative one. Rate of development of the floral bud is dependent upon the intensity of the original act of induction. A measure of the rate of development based upon a series of floral stages has been previously described (21). By the application of this system the quantitative nature of induction can be demonstrated by the relationship between length of the inductive dark period and subsequent rate of floral development, as measured by floral stage a number of days after induction (this is illustrated by the control points of the experiment shown in fig 4). The longer the dark period, the more rapidly the buds develop. This may be interpreted on the supposition that the rate of bud development is dependent upon the amount of flowering hormone produced, and that longer dark periods result in the production of more flowering hormone. This view is supported by the fact that buds develop at different rates when the leaves are removed from Xanthium plants at different times after the beginning of induction (9, 21, 22, 23). If leaves are removed immediately following induction the plants seldom flower. If leaves are removed after a sufficient time, however, floral buds develop at a rate almost as great as that attained by control plants with leaves. Intermediate rates of floral development characterize plants whose leaves are removed at intermediate times (this is illustrated by the points labeled "leaf removed" in fig 5). These results are in accord with the hypothesis that rate of floral development is determined by the amount of flowering hormone which reaches the growing point. The longer the leaves remain on the plant, the more hormone is translocated from them to the growing point.

The experiments below concern kinetic studies on the reactions of the dark period. *Xanthium pensylvanicum* Wall.[4] plants were treated at various times by red light interruption of the dark period and/or the application of auxin (which inhibits the act of induction, 21). The effects of these treatments on floral induction were measured in terms of rate of subsequent floral development, which is assumed to measure the amount of flowering hormone exported from the leaf.

METHODS

Plants were grown as previously described (3, 20, 21) from seed and maintained in a vegetative condition by daylight supplemented with incandescent irradiation of approximately 100 fc to make up a total day length of approximately 20 hours. To facilitate auxin treatment and to insure controlled light intensity during interruption of the dark period, plants were defoliated to a single leaf after first being classified according to the size of the most rapidly expanding leaf, the one most sensitive to induction. In the experiments reported below, the plants were defoliated to the most rapidly expanding leaf and given a single dark period, unless stated otherwise in the figure headings. A weak green light (ca 2 μw/cm^2 for 10 minutes at 2-hour intervals) was used to facilitate treatment during the dark period.

The growing points of treated plants were examined under a dissecting microscope (36 diameters magnification) approximately 9 days after induction. Rate of floral development as measured by the pres-

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[4] Synonymous with *X. saccharatum*, the name used by various other workers in photoperiodism. Specimens of the type of plants used in these studies have been filed by K. C. Hamner at the herbarium of the University of California at Los Angeles.

ent stage system is usually constant with time over this period (20, 21). The appropriate time for dissection was determined by examining controls at various times after induction.

Red light interruption of the dark period was obtained by placing a group of plants under an air-cooled box containing 6 incandescent 300-watt bulbs placed 6 inches above red filters. The intensity at the leaf level (approximately 10 inches below the filters) was 1,800 $\mu w/cm^2$ of red light. The plants were adjusted under the lights so that the level of the leaves would be approximately the same.

Naphthaleneacetic acid (NAA) was used as the auxin unless stated otherwise. When leaves were dipped in auxin solution (NAA dissolved by addition of KOH and then adjusted to pH 7.0; containing 2 drops of Tween 80 wetting agent per liter) at the beginning of induction and allowed to dry, it was found that a 2.0×10^{-4} M NAA solution inhibited flowering by about 50 % (induction by a single 16-hr night). A second method of auxin application consisted in dipping plants in auxin solution and then rinsing them after a measured interval of time. A rinse with tap water was followed by a rinse with distilled water. In this case a 30.0×10^{-4} M NAA solution left on the leaf 10 minutes inhibited flowering after a 16-hour night by about 50 %. The second method is the most desirable since it defines exactly the time of auxin penetration into the leaf (20).

The application of auxin by any method increases by a considerable factor the variation of floral stages within a given group of treated plants. This appears in the data as a poor fit of points to the smooth curves drawn in the figures presented below as compared to the data of experiments or treatments in which auxin was not applied.

Results

Effect of Red Light Interruption during the Dark Period: Although previous workers have investigated the kinetics of the low intensity light reaction (4, 5, 8, 15, 19, 24, 25), the data available record only the presence or absence of flower buds and hence cannot be considered quantitatively. In order to obtain quantitative data on the effectiveness of various red light interruption treatments, groups of plants were irradiated as described above for brief intervals (from 10 sec to 10 min in different experiments) at various times during an inductive dark period. Figure 1 gives the results of an experiment in which the inductive dark period was either 12 or 16 hours and the light interruption was for 60 seconds. The data of figure 1 show the following: 1) Red light interruption 8 hours after beginning of the dark period completely inhibits induction regardless of the length of the dark period. 2) Red light interruption after 8 hours of darkness, in this experiment is equivalent to returning the plants to continuous light. The second halves of both curves of figure 1 are identical with the curves which are obtained by plotting length of inductive dark period against floral stage. 3) The

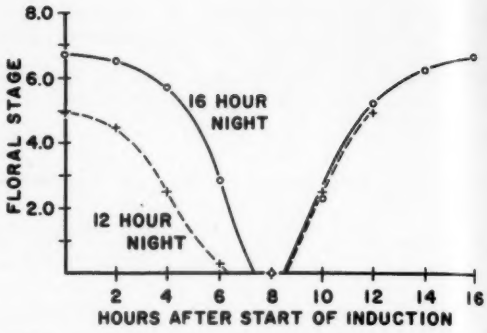

Fig. 1. The effect of red light interruption (60 sec) upon floral induction as a function of time of application during a 12- or 16-hr dark period. May 4, 1954—14 plants per treatment, dissected after 9 days.

first part of each curve is approximately that expected on the basis that the period of uninterrupted darkness following the light interruption alone functions as the dark period. The longer the period of remaining darkness, the higher the floral stage (the more flowering hormone produced in the leaf). 4) The symmetry of the two halves of each curve is, however, not perfect. Darkness following a light interruption is somewhat more effective in induction than darkness preceding a light interruption. This effect is lessened as the 8-hour point is approached.

Observations 1, 3 and 4 have been confirmed in 5 separate experiments. In certain experiments, however, observation 2 does not hold. Darkness near the end of an extended dark period may inhibit induction to some extent. This effect may be especially pronounced when the darkness follows a light interruption. This is illustrated in figure 2. Plants were treated as in the experiment of figure 1 with brief light interruptions (12 sec) during dark periods of

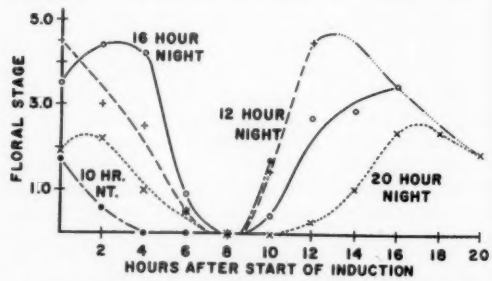

Fig. 2. The effect of red light interruption (12 sec) upon floral induction as a function of time of application during 10-, 12-, 16- or 20-hr dark periods. July 28, 1954—10 plants per treatment, dissected after 9 days. The line connecting the control points of the 12-, 16- and 20-hr nights shows the relationship between floral stage and length of the dark period, and shows the destruction of flowering hormone which takes place near the end of extended dark periods even without light interruption.

various lengths (10, 12, 16 or 20 hrs). In this experiment dark periods of longer than 13 hours were less effective than shorter dark periods. If a light interruption is given after 13 hours of darkness, the resultant inhibition of flowering is proportional to the remaining period of darkness (except for the 16- and 20-hr interruptions of the 20-hr night). Furthermore, interruptions given near the beginning of the 16- or 20-hour dark periods actually increased the degree of flowering over that of the non-light interrupted controls. It would appear that these early interruptions increase hormone production by shortening the effective period of darkness and thus reducing the excess over the critical 13-hour period.

The inhibitory effect of prolonged darkness appears to be identical with that reported by Lockhart and Hamner (16). Thus the curves of figure 2 may be interpreted by the hypothesis that a destruction of flowering hormone may take place in an excessively long inductive dark period. Why this effect may be noted in some experiments and not in others is not clear. The destructive effect may, however, be demonstrated most easily in experiments in which the single remaining leaf is younger than the most rapidly expanding one (20). The plants used in the experiment shown in figure 2 were somewhat younger than those used in the other experiments reported herein, but the physiological age of the one leaf left on each plant was the same in all experiments.

The 16- and 20-hour curves of figure 2 have a somewhat cyclic form similar to those obtained by Wareing (24) and by Carr (5). It is possible that the above supposition relating to a destruction of flowering hormone in prolonged darkness may also apply to the results of these workers. A critical test of this possibility would require the study of floral development after dark periods which are multiples of 24 hours.

The same general relations between time of red light interruption and subsequent rate of floral development were obtained regardless of the length of the red light interruption. To measure the amount of light energy required to produce the maximum interruption effect, groups of plants were irradiated for varying lengths of time (2, 4, 6, 12 and 60 sec) at various times after the beginning of a 16-hour dark period (3.25, 5.75, 8 and 12 hrs). The results are given in figure 3. It is evident that the maximum inhibition of floral induction elicited by light interruption is strongly dependent upon when the interruption is given, as has already been shown by the data of figures 1 and 2. The amount of light required to produce this maximum effect appears, however, to be constant regardless of when the interruption is given. Parker et al (19) report that the product of light intensity and period of exposure to light needed to produce a given amount of inhibition of flowering in Xanthium is constant. The amount of light energy required to produce a given amount of inhibition at any given time may therefore be calculated from the present data since the intensity remains constant. The data of figure 3 indicate that maximum response

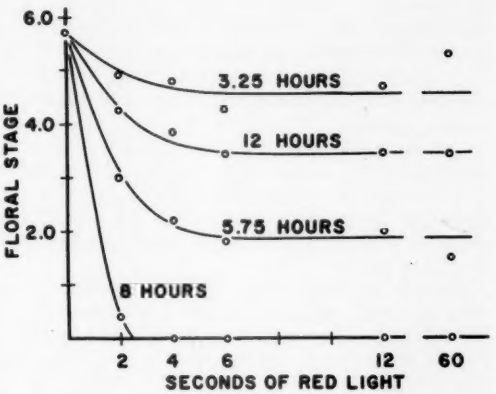

FIG. 3. Relation of floral stage to the duration of a red light interruption given at various times during a 16-hour dark period. July 26, 1954—10 plants per treatment, dissected after 9 days.

was obtained by exposing the plants to periods of more than 6 seconds of red light. Since the intensity of the red light was approximately 1,800 $\mu w/cm^2$, it follows that approximately 10,000 $\mu w/cm^2$ of red light are sufficient to produce maximum response at any time more than 3.25 hours after the beginning of the dark period.

Borthwick et al (4) present evidence that the low intensity light reaction consists of a conversion of a red-receptive form of a photo-receptor pigment to a far-red-receptive form of this pigment. They suggest further that flowering hormone production does not take place unless the pigment is in the red-receptive form; that this pigment is in the far-red-receptive form at the beginning of the dark period (due to exposure to red wave lengths during the day); and that the pigment undergoes a spontaneous conversion to the red-receptive form in the dark. This suggests the possibility that the critical night length is controlled by the time required for the spontaneous conversion of far-red-receptive to red-receptive pigment to take place. It is possible that the amount of light required to produce a maximum red light interruption effect may be a measure of the amount of photoreceptor pigment present in the red-receptive form. The data of figure 3 interpreted on this basis indicate that conversion of far-red-receptive to red-receptive pigment takes place before 3.25 hours after the beginning of induction. Borthwick et al (4) found it possible to shorten the critical night length about 2 hours by irradiating plants with far-red light just before the beginning of the dark period. This finding is in agreement with the above conclusion, and with the conclusion of Withrow and Withrow (25) based upon experiments of a completely different nature. It follows that far-red light should have no effect upon the critical night length when given more than about two hours after the beginning of induction.

EFFECTS OF APPLIED AUXIN: It will now be shown that the effect of applied auxin upon the photoperi-

odic response is not due to any effect upon the critical night length but consists in a net reduction in the amount of flowering substance transported from the leaf. Groups of plants were treated with various concentrations of auxin and then with dark periods of various lengths. The results of 3 such experiments are given in figure 4. Applied auxin has little or no effect upon the critical night length, but the degree of flowering (net amount of flowering hormone produced) of plants given longer than the critical dark period is reduced in proportion to the concentration of applied auxin.

To test the effect of the time of auxin application upon flowering, groups of plants were treated as described above with various concentrations of auxin at various times before, during and after an inductive dark period. The data of figure 5 are typical of 10

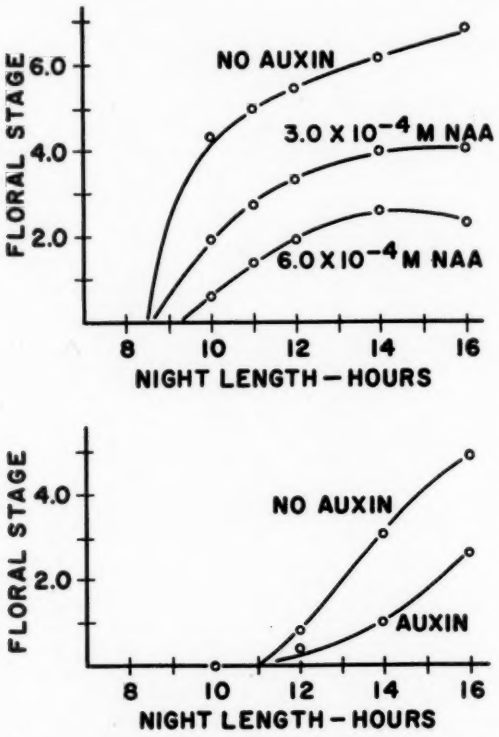

FIG. 4. The relation of flowering to length of the dark period and to applied auxin. *Upper figure.* For plants with leaves dipped in 3.0×10^{-4} M NAA. March 11, 1954—20 plants per treatment, dissected after 13 days. For plants receiving 6.0×10^{-4} M NAA: March 1, 1954—20 plants per treatment, dissected after 10 days. The stages were adjusted so that controls matched the controls of the 3.0×10^{-4} M treatment. *Lower figure.* May 26, 1953—20 plants per treatment, dissected after 17 days, 1.5×10^{-4} M NAA sprayed on the leaves; plants defoliated to the next leaf older than the most rapidly expanding one.

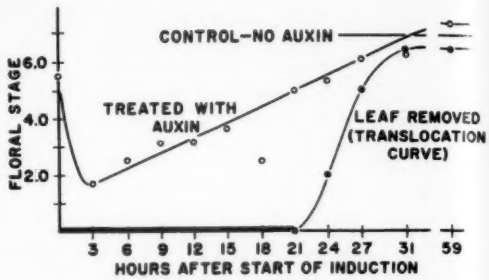

FIG. 5. Effect of auxin in inhibiting floral induction as a function of time of application. April 10, 1954—16 plants per treatment, dissected after 13 days, leaves dipped in water, then after 5 min in 20.0×10^{-4} M NAA, and then after 10 min rinsed in tap water followed by distilled water.

such experiments. It is apparent that auxin becomes increasingly more effective in floral inhibition as it is applied later and later during the first two or three hours after the beginning of the dark period. Auxin applied after this time becomes less and less effective until finally it is completely ineffective. Auxin applied still later increases rate of bud development slightly (21). It is evident also that the slope of the curve relating rate of floral development to time of auxin application shows no sharp change at the end of the dark period. The data show considerable scatter, but no consistent deviation has been detected in different experiments,[5] and a smooth curve appears to best describe the actual kinetics of the auxin reaction. Finally, the time at which auxin becomes ineffective in floral inhibition is essentially the time at which the concentration of the flowering substance has been built up to maximum level outside the leaf, as shown by defoliation experiments (explained above). From experiments with defoliation at varied times after various night lengths (not shown in fig 5), night length seems to be relatively unimportant in determining time of translocation of the flowering stimulus from the leaf and hence in determining the time at which applied auxin becomes ineffective in floral inhibition. Time of translocation is, however, affected strongly by time of year due perhaps to differences in light intensity. Translocation was found to be half completed in 25 hours after the beginning of induction in July but only in 40 hours in December.

The relationship between inhibition of flowering by red light interruption and inhibition by applied auxin was studied with plants which were irradiated with red light or treated with auxin or both at various times during a 16-hour inductive dark period.

[5] In some experiments, auxin seems to be less effective when applied in the heat of the day. This is probably due to low humidity which results in rapid drying of the auxin solution on the leaves so that there is insufficient time for auxin penetration. This effect may be overcome by raising the humidity and/or using the method in which the auxin solution is rinsed off the leaves after a brief, measured interval.

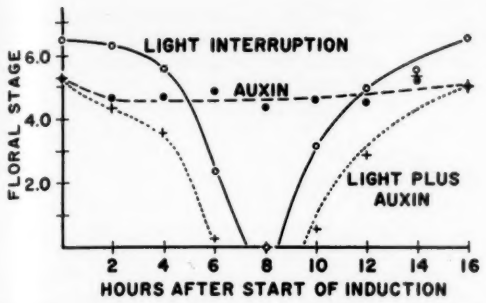

FIG. 6. Inhibition of floral induction by a red light interruption (60 sec), by applied auxin, or by both together, as a function of time of application during the dark period. May 19, 1954—15 plants per treatment, dissected after 9 days. Leaves of auxin-treated plants were dipped first in water, then in 20.0×10^{-4} M NAA, and then rinsed after 5 min as explained in figure 5. The dotted line is theoretical and represents the sum of the inhibitions of the other two curves.

The experiment was repeated three times. In two cases NAA was used as the auxin while in the third case indoleacetic acid (IAA) was used. Figure 6 gives the results of an experiment with NAA. Light or auxin alone acted as in the experiments described above. The inhibition produced by the two factors together is very nearly the sum of the inhibition produced by each factor applied alone.

Discussion and Conclusions

The experimental results of this work may be summarized as follows: 1) A red light interruption of sufficient intensity, given 8 hours after the beginning of induction inhibits flowering completely. 2) Later interruptions are equivalent to returning the plants to full light, although in certain cases darkness following the light interruption inhibits subsequent flowering. 3) The effectiveness of a red light interruption given earlier than 8 hours after the beginning of the dark period depends upon the length of darkness which follows the interruption. 4) The quantity of light energy required to bring about the maximum inhibitory effect of an interruption of the inductive dark period is constant after the first 2 or 3 hours of the dark period. 5) Application of auxin decreases the amount of flowering hormone which reaches the growing point, but has little effect upon the critical night length. 6) Inhibition of flowering is maximal when auxin is applied shortly after the beginning of the dark period. As auxin is applied later, its effectiveness decreases at a nearly constant rate and it is ineffective when the full concentration of flowering hormone has been built up outside the leaf. There is no sharp change in effectiveness of auxin in floral inhibition at the end of the dark period. 7) The inhibitory effect of applied auxin and of red light interruption applied simultaneously are approximately equal to the sum of the inhibitory effects of these agents applied separately.

These results and those of other workers (1, 2, 3, 4, 5, 6, 9, 10, 12, 14, 15, 16, 17, 19, 25) suggest that during the dark period 3 consecutive reactions take place:

a. The spontaneous conversion, in the dark, of the photo-receptor pigment from a far-red-receptive to a red-receptive form. (Pigment Conversion.)
b. Reaction(s) preparatory to hormone synthesis. (Preparatory Reaction.)
c. Synthesis in the leaf of flowering hormone. (Hormone Synthesis.)

The following features are characteristic of these reactions and at the same time help to define them: Critical night length is determined by the first two reactions. For the Preparatory Reaction to proceed to completion at least some of the pigment must be in the red-receptive form, as this reaction is stopped by red light interruption. Since induction can take place under very weak red light (1, 12), however, Pigment Conversion may not have to be complete for the Preparatory Reaction to begin.

When Pigment Conversion is reversed by a red light interruption, the Preparatory Reaction is also reversed and must begin again, although Hormone Synthesis is only halted but not reversed. If the Preparatory Reaction is stopped (by light interruption) just before the beginning of Hormone Synthesis (near to the critical night length), it may not be resumed regardless of the length of the dark period which follows (5, 6, 24). It seems probable that this may be due to a depletion of energy substrates built up by photosynthesis in the previous light period. Indeed, Liverman and Bonner (14) obtained flowering in plants exposed to a long night interrupted at 3-hour intervals by a flash of light and then given a long period of uninterrupted darkness, providing sucrose or other energy substrates were supplied to the leaves.

The first two reactions seem to be rather insensitive to applied auxin since auxin affects the critical night length only slightly if at all. Net rate of Hormone Synthesis is, however, strongly depressed by applied auxin.

Rate of flowering Hormone Synthesis is a function of the number of hours by which the dark period exceeds the critical length. This rate is rapid for the first 2 hours or so, then tapers off and finally reaches zero after 4 to 8 hours (12 to 16 hrs after the beginning of the dark period).

Under some conditions a destruction of flowering hormone in the leaf appears to take place during the later hours of a prolonged dark period. This produces an apparent cycle of sensitivity to red light interruption.

Applied auxin has been shown to be without effect on critical night length. The data presented here also cannot be interpreted on the basis that auxin inhibits the translocation of flowering hormone from the leaf. Auxin is particularly effective during the period of Hormone Synthesis and translocation seldom begins until the plants are returned to light (9, 20, 21, 23).

In addition, antiauxins which, if applied before induction, overcome the inhibitory effect of auxin, do not counteract the inhibitory effect of auxin if applied after induction as might be expected if the effect were only upon translocation (20). It seems more probable at present that auxin brings about a destruction of flowering hormone in the leaf (13, 22). This idea is compatible, for instance, with the fact that there is no sharp change in effectiveness of applied auxin at the end of the dark period. Further, the reduction in net rate of Hormone Synthesis by applied auxin may be a destruction of a fraction of the flowering hormone normally produced during Hormone Synthesis.

We do not know, of course, how auxin could result in the destruction of a hormone which has been synthesized in the leaf. It appears possible that auxin might initiate other energy-using processes (e.g. related to growth), and that these processes might utilize energy or materials which would otherwise have been used to maintain the flowering hormone in the leaf. These matters remain to be elucidated.

It appears possible that the native auxin of Xanthium is relatively unimportant in the reactions of the dark period. Experiments with applied antiauxin usually show only a slight promotive effect upon flowering, and often this effect may be detected only when the plants receive an inductive dark period under conditions close to the critical (1, 12). Thus application of antiauxin may spare a certain amount of flowering hormone destruction, but since this amount is slight, the amount of auxin normally present may be slight compared to the amount applied in the above experiments.

Summary

1. The reactions of the photoinductive dark period in Xanthium may be studied kinetically by assuming that rate of development of the floral bud (measured by floral stage) is a function of the amount of flowering hormone produced during induction. Evidence in support of this assumption is based upon the varying rate of floral development of plants and induced by various night lengths, or after defoliation at various times following induction (during translocation of the flowering hormone).

2. Red light interruption of the dark period is most effective approximately 8 hours after the beginning of induction. The effectiveness of light interruption before this time depends primarily upon the length of the remaining dark period. After 8 hours, light interruption of the dark period may be equivalent to returning the plants to full light, although in some experiments a destruction of flowering hormone takes place in the darkness which follows this interruption.

3. A constant amount of red light is required to bring about maximum inhibition of flowering for any given time after the first 2 or 3 hours of darkness. This may indicate that a red-receptive, photo-receptor pigment has reached its maximum concentration within the first 2 or 3 hours of darkness.

4. Auxin applied at the beginning of induction may strongly affect the amount of flowering hormone subsequently produced, but has little effect upon the critical night length.

5. Applied auxin is most effective in inhibiting floral induction when applied 2 or 3 hours after the beginning of the dark period. After this time its effectiveness decreases gradually until it becomes ineffective some hours after the plants have been returned to light. This is also the time required for a maximum concentration of flowering hormone to be built up outside the leaf by translocation. The slope of the curve relating floral stage to time of auxin application shows no sharp change at the end of the dark period.

6. The inhibitory effects of light and of auxin applied together equals the sum of the inhibitions caused by each agent applied alone.

7. It is suggested that three reactions take place during the dark period. a. Conversion of photoreceptor pigment from a far-red to a red-receptive form (complete in 2 or 3 hours), b. Preparatory Reaction(s), which together with Pigment Conversion determine the critical night length, c. Actual Hormone Synthesis.

8. It is suggested that applied auxin may bring about a destruction of flowering hormone in the leaf. The native auxin of the leaf may participate in the flowering response in a similar way, although it is ordinarily present in concentrations too small to be of great importance.

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INDUCTION OF FLOWERING IN LONG DAY PLANTS BY APPLIED INDOLEACETIC ACID[1,2]

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In short day plants the promotion of flowering by photoinductive cycles may be nullified by the application of auxin (2) and by interruption of the dark period with red light (17). Both of these inhibitory effects can be reversed by the application of an anti-auxin (1, 2, 12). On the other hand light interruption of the dark portion of the photoinductive cycle promotes floral induction in long day plants (3, 8). On the basis of these results we might expect either that the flowering of long day plants would be inhibited by auxin applied during photoinduction, or that auxin would replace or supplement low intensity light and actually cause flowering of plants kept in a short day regime. The experiments reported in this paper were undertaken to try to discover the role of auxin in the flowering of long day plants. The results demonstrate for the first time that auxin given in addition to low intensity light may cause flowering of long day plants under conditions in which the non-auxin treated controls remain vegetative.

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Materials and Methods

Two long day plant species, *Hyoscyamus niger* (annual variety) and *Silene Armeria*, were used for the experiments reported here. Experiments with a third long day plant, *Crepis capillaris*, also indicate a flower promoting effect of auxin applied to plants grown under threshold conditions. This material, however, proved so variable, that the experiments, taken by themselves, would not be conclusive and are not presented here. The photoperiodic response of Hyoscyamus was studied by Lang and Melchers (9). The action spectrum for the light flash reaction in this species was determined by Parker and co-workers and was found to be identical with the photoperiodic action spectrum of other day-length-dependent plants of both the long day and short day types (16). The strain used in our experiments is the same as that used by earlier workers (annual yellow flowered variety). The photoperiodic behavior of Silene, a species not previously used for photoperiodic work, was studied by Liverman and Lang (12, 14) and was found to be typical for a long day plant. This strain[5] was collected in the yard of one of us (J. L.) in San Gabriel, California where it had grown spontaneously for a number of years. A majority of the experiments reported below were carried out in the con-

[5] Seed of this species may be obtained from the Vaughan Seed Company, 601 W. Jackson Blvd., Chicago, Illinois.

trolled environmental conditions of the Earhart Plant Research Laboratory (19). Hyoscyamus was grown in a gravel-vermiculite mixture, Silene in a gravel-sand-vermiculite mixture. All plants were watered daily with Hoagland's nutrient solution.

Experiments with both species were conducted under threshold conditions consisting of a basic 8-hr period of high intensity light given either in the greenhouse or in rooms lighted by panels of fluorescent and incandescent lamps followed by 16 hours of supplementary light of different intensities. In the early experiments the variations in intensity of the supplementary light were achieved by placing the plants at different distances from a 15-watt incandescent bulb suspended well above the level of the plants. In some later experiments with Hyoscyamus the plants were given supplementary light in individual rooms where the intensity was adjusted with the aid of variable voltage regulators (powerstats). In all experiments each container of plants at a given intensity station was shifted daily according to a schedule to insure uniform supplementary light treatment of all plants at that station. This light regime was selected since it is to be expected that the effects of various experimental treatments on the photoperiodic response become most apparent if treatment is applied under conditions wherein the plants are just below or just above the boundary which separates inductive from non-inductive photoperiods, or flowering from non-flowering. The intensity of the supplementary light was measured with a photometer which had been calibrated against a standard lamp. In the experiments with the powerstats possible differences in spectral characteristics of the light were not considered.

Immediately after the end of the 8-hr period of high intensity light the leaves were thoroughly wetted by spraying with various concentrations of indoleacetic acid (IAA) containing approximately 0.001 % Atlas Tween 20 or Tween 80 as a wetting agent.

Tween 20 and Tween 80 are the monolaurate and the mono-oleate respectively of polyoxyethylene sorbitan. The controls were sprayed with water alone containing Tween. New IAA solutions were made every two or three days and were stored in a cold room at approximately 5° C between sprayings.

Experiments with Hyoscyamus niger

The plants used in the first experiment were grown in Earhart Plant Research Laboratory under an 8-hr day until they were 10 weeks old. Prior to the experimental treatment the plants were grown at a temperature of 23° C day and 20° C day–17° C night, being shifted at regular intervals from one temperature to the other. At the end of this growth period the plants were randomized into 40 treatment groups and put under threshold conditions. During the 17 to 25 days of threshold treatment, the plants received high intensity light at 23° C in the greenhouse and were then sprayed daily with various concentrations of IAA before being moved to the darkroom for supplementary light. Five different intensities of supplementary light were used, the plants receiving a total of 218, 71, 34, 20 or 16 fc min/night. After 17 days, all plants receiving the highest level of supplementary light had elongated and their treatment was discontinued. The other series were treated for a total of 25 days. At the end of the experimental treatment those plants which did not show visible buds were returned to the greenhouse for growth under short day conditions for an additional 11 to 19 days prior to being examined for the presence or absence of microscopic flower buds.

Results: All of the control and experimental plants at the two highest intensities initiated flowers with the single exception of 2 of 6 plants at the second intensity which were sprayed with 30 mg/l of IAA. The results for the 3 lowest intensities are presented in figure 1. At these intensities non-auxin treated controls remained vegetative whereas auxin treated plants showed a marked inductive response with 45 to 100 % of the treated plants initiating flowers. The optimum indoleacetic acid concentration in this experiment appears to lie between 3 and 10 mg/l, with 30 mg/l being less effective. The data in table I, in which the number of leaves produced during the experimental period is given, shows that this promotive effect of auxin is quite specific. The plants treated with IAA and induced to flower produced only about one half as many leaves as the plants which did not initiate flowers, thus, auxin did not simply accelerate the growth of the plants, but actually caused the initiation of flowers. Only at the highest auxin concentration does there appear to be a discrepancy to this conclusion. This discrepancy may possibly be attributed to the reduced growth rate of the plants treated with such a high auxin concentration.

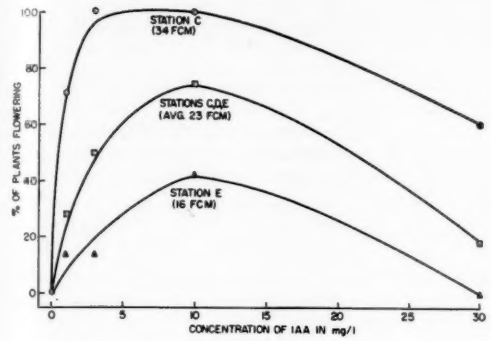
These results were confirmed in all essential respects in four additional experiments. Data of one of these experiments are summarized in figure 2. A comparison with figure 1 shows that the results of the

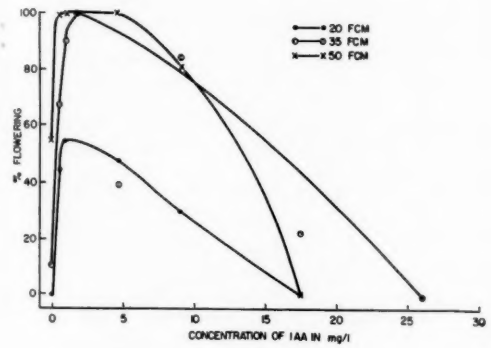
Table I

Average Number of New Leaves Formed by Hyoscyamus Plants from Start of Treatment to Flower Initiation or Dissection

Amt of suppl light, fc min	State	Conc IAA, mg/l				
		0	1	3	10	30
218	Flowering	7.3	7.9	10.4	10.9	11.8
	Non-flowering	...	...	...	...	...
71	Flowering	13.9	13.6	13.9	12.9	15.4
	Non-flowering	...	...	...	...	20.6
34	Flowering	...	17.2	16.5	16.6	19.9
	Non-flowering	25.0	30.5	...	...	19.1
20	Flowering	...	...	...	17.4†	...
	Non-flowering	33.3*	33.0*	23.9**	36.0**†	23.8**
16	Flowering	...	13.0†	17.0†	19.6	...
	Non-flowering	34.8	32.4	30.1	27.1	30.8

* Dissected 11 days after end of treatment.
** Dissected 9 days after end of treatment.
† One plant only.





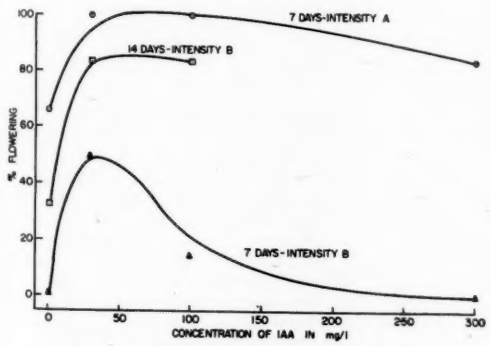


FIG. 1 (*upper*). Floral induction in *Hyoscyamus niger* as a function of light intensity and IAA conc.

FIG. 2 (*center*). Additional experiment showing effect of light and auxin on floral induction in *Hyoscyamus niger*.

FIG. 3 (*lower*). Floral induction in *Silene Armeria* by light and IAA. Plants at station A received approximately 2000 fc min of supplementary light, at station B approximately 400 fc min.

two experiments are quite similar except that the optimum auxin concentration for flower promotion in the last experiment is lower, and that the higher auxin concentrations completely suppress flowering. It was found that the optimum auxin concentration may vary between approximately 3 and 30 mg/l and also that the light intensity at which the auxin effect becomes evident may vary from one experiment to another over an approximately 2-fold range. These differences from experiment to experiment may be related to the age and general condition of the plants. This difference in auxin concentration needed to give the maximum response is particularly noticeable when comparing plants given their high intensity light as artificial light with those receiving their light period in the greenhouse. Plants receiving high intensity light from the artificial source (fig 2) have uniformly given a maximum response at lower auxin concentration than greenhouse grown plants. In any case the important fact is that auxin shows a marked inductive effect in both these experiments as well as in other similar experiments not reported here.

EXPERIMENTS WITH SILENE ARMERIA

The type of experiment reported above for *Hyoscyamus niger* has been completed with another long day plant, *Silene Armeria*. All experiments reported below were conducted in Earhart Plant Research Laboratory under controlled conditions of temperature and light intensity. Prior to the experimental treatment, the plants were grown under a regime of eight-hour days in the greenhouse at 20° C and 16 hours of darkness at 14° C. When the plants were 2.5 months old, they were randomized and placed in a dark room maintained at 23° C. Here they received 450 to 750 fc of light from a fluorescent source plus light from four 75-watt incandescent bulbs from 8 A.M. until 4 P.M. for 3 additional short days during which they were sprayed with IAA or water solutions containing Tween 80. The plants were then placed under threshold conditions and the spraying continued. These threshold conditions consisted of a basic period of high intensity light as described above from 8 A.M. to 4 P.M. and supplementary light from a 15-watt frosted incandescent bulb from 4 P.M. until 8 A.M. One-half of the plants from each treatment group were removed to short day conditions in the greenhouse after 7 days of threshold treatment and the remaining one-half at the end of 14 days. The plants were dissected 30 days from the beginning of the threshold treatment.

RESULTS: The results of the experiments with Silene are shown in figure 3. It is obvious that Silene is not as responsive to low intensity light as is Hyoscyamus since it takes approximately 2000 fc minutes of light for even partial induction. It is also obvious that plants treated with IAA show a marked increase in flowering.

DISCUSSION

So far as the authors are aware this report presents the first evidence that auxin applied to a long

day plant causes flowering under conditions in which the controls remain vegetative, i.e., this is the first evidence for a qualitative effect of auxin on the flowering of a long day plant. It should be noted, however, that at least 3 day-neutral plants, the pineapple, the sweet potato, and the litchi have been induced to flower by auxin application (5, 6, 18). Previous experiments with long day plants, which were carried out by various workers (7, 10, 11), have shown that the flowering of a long day plant may be quantitatively increased by low concentrations of auxin. This effect, however, was observed only under strict long day conditions and it is not clear whether it is specific. No effect of auxin was observed under short day conditions. Only Claes (4) has conducted experiments under semithreshold conditions and even under these conditions no effect of auxin was observed. Our results show that induction by applied auxin becomes apparent only under the very specific conditions wherein auxin has been made the limiting factor. These conditions were achieved by bringing the plants very near the threshold for flowering by exposure to small amounts of supplementary light. Under conditions where neither auxin nor light alone are effective in inducing flowering, the two are able to interact in such a way as to cause flowering. The exact mechanism of this interaction is not known although some, as yet, unverified suggestions have been made (13, 15). A thorough understanding of this interaction should lead us closer to a solution of the mechanism of floral induction.

Summary

It has been shown in 7 separate experiments that 2 long day plants, *Hyoscyamus niger* and *Silene Armeria*, can be induced to flower by the application of IAA to the leaves of plants grown under threshold conditions.

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EFFECT OF BICARBONATE ION ON THE RESPIRATION OF EXCISED ROOTS[1,2]

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Investigations have established the bicarbonate ion as a contributing factor in a plant chlorosis similar to that induced by soils high in calcium carbonate. This type of plant yellowing associated with an inactivation of iron is referred to as chlorosis in this paper. Chlorosis has been induced in susceptible plants by increasing the level of the bicarbonate ion in the growth medium (5, 8). A combination of high pH and high bicarbonate ion concentration has not prevented iron absorption, but apparently has resulted in the inactivation of iron within the plants. Lindsay and Thorne (5) found that nutrient solutions containing high bicarbonate ion concentrations increased the accumulation of iron in the roots of Great Northern bean plants, but decreased the translocation to leaves and stems. Similar results were obtained by Warnock (11).

The bicarbonate ion effect cannot be attributed to high pH alone. Porter and Thorne (6) conducted nutrient culture experiments in which pH and bicarbonate ion concentrations were varied by regulating the carbon dioxide pressure of the aeration stream. Great Northern bean plants developed chlorosis with high bicarbonate ion concentrations regardless of pH. Stewart and Preston (7) found that the bicarbonate ion, more than any other component of a bicarbonate buffer solution, suppressed the uptake of bromide ions by potato disks. Increasing the level of $KHCO_3$ in the solution depressed protein synthesis and oxidase activity.

Plants differ in susceptibility to chlorosis (1, 5, 11). Brown (1) investigated some copper and iron enzymes in a group of plants in relation to chlorosis susceptibility. Plants of the group which become chlorotic when grown on a high-lime soil were resistant to chlorosis when grown on an organic (copper deficient) soil. Plants which were chlorotic when grown on a calcareous soil were much lower in catalase activity than were green plants of other species grown on the same soil or of green plants of the same species grown on different soils. Ascorbic acid oxidase appeared to be a good index of available copper in most plants whether the plants did or did not show visual copper deficiency symptoms.

Since the known functions of copper and iron in plants are associated solely with enzyme systems, the difference in behavior of chlorosis-susceptible and chlorosis-resistant plants indicates differences in the relative dependence of these plants on iron and copper enzyme systems. Kadry (4) has noted a pronounced accumulation of bicarbonate-derived carbon in several plants. The pronounced effects of the bicarbonate ion on chlorosis-susceptible plants further suggests that the principal bicarbonate effect may be on iron enzyme systems and that copper systems would be affected to a much lesser degree.

MATERIALS AND METHODS

Seeds of the plants to be studied were treated in a 0.5 % sodium hypochlorite solution, rinsed in distilled water, and allowed to soak for two hours. The seeds were then placed on water-saturated, absorbent pads, covered with sterile paper towels and incubated at a constant temperature until roots had obtained a length of 2 to 3 cm. Apical centimeter sections were excised from the roots and placed in Warburg flasks containing 2 ml of Hoagland's base nutrient solution plus additional solutes as indicated. Respiration measurements using the standard Warburg manometric technique were begun as soon as constant temperature conditions were established. After each run the roots were placed in tared crucibles and dried. Respiration rates were calculated on a dry weight basis and are expressed as μl of O_2 absorbed per mg of dry weight per hour. The pH of each nutrient solution was determined after completion of the respiration measurements by use of phenol red and thymol blue indicators.

In a preliminary study it was found that the oxygen uptake rate of Great Northern bean roots between the 48- and 96-hour age period is fairly constant. Roots of this age period were used for subsequent work. The apical centimeter of the roots was selected for use because experimental work showed it to have the greatest respirational activity. For bean roots (96-hours after first sprout appeared from germinating seed), the average rate of oxygen consumption per hour per milligram of dry tissue for the 1st, 2nd, and 3rd centimeters of root tips was 13.1, 9.6, and 5.7 μl, respectively.

EXPERIMENTAL RESULTS

THE EFFECT OF COMMON SOIL IONS ON THE RESPIRATION OF EXCISED BEAN ROOTS: Under field conditions the chlorosis being investigated occurs on soils containing large amounts of $CaCO_3$. The disease is accentuated by keeping the soil moist. Under such situations the two principal ions in the soil solutions are Ca^{++} and HCO_3^-. Experiments were, therefore, planned to compare the effects of Ca^{++} with Na^+ and of HCO_3^- with Cl^- and SO_4^{--} ions, on the uptake of oxygen by the roots.

Hoagland's nutrient solution was used as the standard solution in all flasks. In four Warburg flasks the basal solution was supplemented with 25 meq/l of either $NaHCO_3$, NaCl, $CaCl_2$ or Na_2SO_4. A control flask contained only the basal solution. Since the solutions containing bicarbonate ions increased in pH

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TABLE I

THE INHIBITION OF OXYGEN UPTAKE BY EXCISED BEAN ROOTS AS AFFECTED BY Na, Ca, HCO_3^-, Cl^- AND $SO_4^=$ IONS AT 25 MEQ/L IN HOAGLAND'S SOLUTION

SALT	ION COMPARISON	HRS AFTER TREATMENT					
		1	2	3	4	5	6
		% Difference in oxygen uptake					
Basal solution *—NaCl		5.0	4.3	2.8	8.0	5.2	1.8
Basal solution *—$NaHCO_3$		0.0	−10.5 **	−14.9	−24.6	−27.6	−25.0
NaCl—$NaHCO_3$	Cl^-—HCO_3^-	−4.9	− 6.0	−11.1	−18.5	−24.2	−23.0
NaCl—$CaCl_2$	Na^+—Ca^{++}	0.0	0.0	− 3.3	0.0	0.0	− 1.8
NaCl—Na_2SO_4	Cl^-—$SO_4^=$	−6.7	+ 4.1	+ 1.6	+ 6.3	+ 3.4	+ 4.7

* The average rate of oxygen uptake in the basal solution (Hoagland's solution) cultures was 17.6 μl/hr × mg of dry tissue.

** A negative value indicates oxygen uptake is the corresponding percentage less for the second treatment than for the first treatment in each pair.

during the experimental period, KH_2PO_4 was added at 10 meq/l to each flask as a buffer. The $CaCl_2$, NaCl, Na_2SO_4 and control solutions were adjusted to an original pH of 8.8; $NaHCO_3$ solutions were adjusted to 8.5. Eight apical centimeter sections of bean roots were placed in each flask. Two flasks contained $NaHCO_3^-$ nutrient solutions; the four remaining flasks contained the other salt-nutrient solutions. The average rates of oxygen consumption were determined in three separate experiments. The percentage differences in oxygen uptake for roots in different pairs of solutions are tabulated in table I.

During the first hour bicarbonate induced no pronounced inhibition of oxygen uptake. Subsequent inhibition by bicarbonate ion seemed to increase with time; the fourth, fifth, and sixth hours showing a marked decrease in respiration over the previous hours. Calcium, sodium, chloride, and sulfate ions produced less inhibition of oxygen uptake than did bicarbonate. The salts, other than $NaHCO_3$, caused no significant inhibition. The osmotic pressures of the nutrient solutions used in this experiment varied from 1.5 to 1.8 atmospheres, and differences in osmotic pressure were not related to the inhibitions observed.

RESPIRATION OF EXCISED BEAN ROOTS AS RELATED TO PH: When bicarbonate ion was added to a solution and this solution was used in micro-respirational studies, the pH of the solution rose as much as 0.5 pH unit during the course of the experiment, even though the solution was buffered with 10 meq/l of KH_2PO_4. It might be argued that the inhibitory effect of the bicarbonate ion resulted from the high pH of the solution. An experiment was designed to check this.

Hoagland's nutrient solution was buffered with 10 meq/l of KH_2PO_4. Solutions were brought to the desired pH with NaOH and HCl. Each flask contained the same nutrient solution, differing only in pH. Excised root sections were prepared for study as before, and their oxygen uptake was followed for a period of 3 hours. The results of these studies are shown in table II.

Oxygen uptake was fairly constant over the pH range 4.5 to 9.0. There was definite inhibition at pH values of 3.0 and 10.5. The results indicate the inhibition by bicarbonate ion observed in previous experiments was the result of factors other than pH.

As a further check on pH effects, seedlings with roots two centimeters long were placed for 16 hours in Hoagland's solution buffered at pH 9.4. The apical centimeter sections of these roots were then excised and immersed in Hoagland's solution, pH 8.8, contained in Warburg flasks. The oxygen uptake of these roots was compared to that of roots germinated on pads at a slightly acid pH. The roots from seedlings treated with pH 9.4 solutions absorbed oxygen at only slightly slower rates than roots from control seedlings. The slight inhibition manifested from exposure to the high pH solution could not account for the inhibitions observed in the presence of bicarbonate ions. Excised roots in a bicarbonate solution for 6 or 7 hours suffer a 60 to 70 % decrease in respiration. These results further indicate that the bicarbonate ion has a specific effect on the respiration distinct from the pH effect.

THE EFFECT OF THE BICARBONATE ION ON EXCISED ROOTS FROM VARIOUS PLANTS: This phase of respirational studies covered the effects of bicarbonate on excised roots from various plants.

The seeds were treated and germinated as previously outlined (we are indebted to Dr. J. C. Brown of

TABLE II

OXYGEN UPTAKE OF EXCISED BEAN ROOTS AT DIFFERENT PH LEVELS AS A PERCENT DIFFERENCE FROM UPTAKE AT PH 7.5

PH START	PH FINISH	HOURS AFTER START			AV DIFF AS % UPTAKE AT PH 7.5
		1	2	3	
		Difference as % of uptake at pH 7.5 *			
3.0	3.0	−29.2	−57.2	−64.0	−51.5
4.5	4.5	+ 3.5	+ 7.6	0.0	+ 1.3
6.0	6.0	+ 9.9	+ 6.4	+ 9.5	+ 8.9
9.0	8.5	+ 4.2	+ 3.3	+ 2.5	+ 3.4
10.5	9.3	− 5.8	−11.7	−19.0	−11.8

* The average rate of oxygen uptake at pH 7.5 was 14.6 μl/mg of dry tissue.

the A.R.S., Beltsville, Maryland, for supplying seeds of plants used in his studies). Eight to ten of the larger excised roots, such as those of corn, peas, and beans were placed in each respiratory flask. When the smaller excised roots of tomato, wheat, and rye were used, 20 or more were used per flask. The solutions used in each series of studies were as follows:

Hoagland's solution	pH 7
Hoagland's solution—15 meq/l $NaHCO_3$	pH 8.0
Hoagland's solution—20 meq/l $NaHCO_3$	pH 8.2
Hoagland's solution—25 meq/l $NaHCO_3$	pH 8.5

The solutions were not buffered to the same pH because previous experiments indicated that pH effect is slight over the pH range encountered. The flasks containing the nutrient solutions differed only in pH and meq/l of $NaHCO_3$. The results in Table III show the effect of bicarbonate ion on the oxygen uptake of the plant species studied. In most cases the higher bicarbonate levels gave the greatest inhibition.

Bean, pea, nasturtium, milo, lupines, and soybeans showed marked decrease in respiration in the bicarbonate solutions. All of these plants except the milo and hawkeye soybean are susceptible to lime-induced chlorosis. The rate of oxygen uptake was not constant throughout the three- or four-hour period, but decreased with time. The inhibition of respiration was in most cases greater at the higher bicarbonate levels and increased with each hour of the experiments. Corn, tomato, spinach, wheat, barley, rye, and okra were affected only slightly by the bicarbonate ion. None of these plants commonly shows chlorosis on the calcareous soil used. Also the roots of these plants displayed no significant increase in inhibition with increased bicarbonate concentration nor did inhibitions increase with time. The effects of bicarbonate were thus quite different for the two groups of plants, and with the exception of Midland milo there was good agreement between bicarbonate effects and plant susceptibility to iron and copper deficiency symptoms.

TABLE III

BICARBONATE INHIBITION OF OXYGEN UPTAKE BY EXCISED ROOT TIPS OF VARIOUS PLANTS

PLANT	NO. OF EXPTS	CHLOROSIS SYMPTOMS ON CALCAREOUS SOIL **	COPPER DEF SYMPTOMS ON ORGANIC SOIL **	% INHIBITION DURING 3RD HR *
Great Northern Bean	4	+	0	23.5
Nasturtium	1	+	...	18.6
Midland Milo	2	0	+	16.7
White Lupine	2	+	0	16.7
Blue Lupine	3	+	0	14.7
P. I. Soybean	2	+	0	14.0
Morse's Progress Pea	1	+	...	11.8
Yellow Lupine	2	+	0	11.2
Hawkeye Soybean	2	0	0	11.0
Ottawa Select Spring Rye	3	0	0	6.8
Thatcher Wheat	4	0	+	6.3
Hannchen Barley	2	0	+	5.7
Utah Hybrid Corn	3	0	...	4.6
Prickly Seeded Spinach	1	0	...	3.6
Marglobe Tomato	1	0	+	1.0
Long Green Okra	1	0	+	1.0

* Average % inhibition of 15, 20, and 25 meq/l $NaHCO_3$ combined over that of low-bicarbonate control.

** From data of Brown (1) and authors results with the same calcareous soil.

Brown (1) observed that catalase activity was lower in lime-induced chlorotic plants than in normal green plants. Ascorbic acid oxidase in the chlorotic plants was about the same as in normal plants. The fact that chlorosis-resistant plants are often sensitive to copper deficiency suggests the presence of alternative copper oxidative enzyme systems that can maintain at least near normal metabolism under conditions of iron stress in many chlorosis-resistant plants.

The bicarbonate-sensitivity of milo coupled with its resistance to lime-induced chlorosis in these tests and sensitivity to copper deficiency, makes it an exception to the relationships noted. However, this milo has been frequently reported to be chlorotic on calcareous soils in Kansas and Arizona even though it was not chlorotic under the conditions of Brown's experiments (1). Also Brown reported a high endogenous activity in determining ascorbic oxidase activity in copper deficient milo plants and a poor correlation between visual deficiency symptoms and ascorbic oxidase activity. These observations led him to conclude that milo appears to be limited in growth through a copper system, but not necessarily in the same manner as wheat and barley.

INHIBITION WITH CARBON MONOXIDE: The previous experiments indicated that bicarbonate ions have greatest effect on root respiration of plants having apparently iron-dominated respiratory systems. Iron carbonyls are decomposed by visible light of short wave length, but copper carbonyls are light stable. Carbon monoxide inhibition provides a simple method, therefore, for differentiating between copper and iron terminal oxidases and might serve as a tool in clarifying the effects of bicarbonates.

The effect of CO on excised roots of bean (chlorosis-susceptible) and corn (resistant to chlorosis) in relation to the bicarbonate ion effect is reported here. The volume ratio of N_2/O_2 or CO/O_2 was calculated in the gas phase. Two partly immersed beakers containing 200-watt bulbs were placed in the constant temperature bath to supply the lighting for the system. Vented flasked-manometers were joined in series and flushed with two liters of the desired gas mixture. The treatments were as follows:

Hoagland's solution	19:1—N_2/O_2
Hoagland's solution	19:1—CO/O_2
Hoagland's solution + 25 meq/l $NaHCO_3$,	19:1—N_2/O_2
Hoagland's solution + 25 meq/l $NaHCO_3$,	19:1—CO/O_2

The cultures were illuminated during the second and third hour. There was no illumination during the first and after the third hours. Average results of several tests are shown in table IV. The 19 : 1 CO/O_2

TABLE IV

THE INHIBITORY EFFECTS OF CO AND $NaHCO_3$ ON THE RESPIRATION OF BEAN AND CORN ROOTS

TREATMENT	PLANT	NO. OF EXPTS	HRS AFTER TREATMENT			
			1	2	3	4
			% *Inhibition*			
CO *	Bean	6	39	16	28	52
CO	Corn	4	47	22	26	58
$NaHCO_3$ **	Bean	6	43	38	36	38
$NaHCO_3$	Corn	4	18	19	14	3
$NaHCO_3$ + CO	Bean	6	52	30	50	67
$NaHCO_3$ + CO	Corn	4	55	30	32	52

* CO : O_2 ratio = 19 : 1.

** $NaHCO_3$ concentration = 25 meq/l.

Treatments added to root tips in Hoagland's solution cultures were illuminated during the second and third hours.

mixture as compared to 19 : 1 N_2/O_2 mixture inhibited bean root respiration 39 % in the first hour. Upon illumination the inhibition was reduced until during the first hour of illumination less than 16 % inhibition was exhibited. The inhibition increased again in the fourth hour when there was no illumination. Corn root respiration was inhibited similarly by CO, and light greatly reduced this inhibition. The respiration of excised bean roots was inhibited 52 % in the first hour by CO + $NaHCO_3$ treatment. Light partially reversed this inhibition. During the fourth hour the inhibition increased to 67 %. The respiration of excised corn roots was inhibited similar to bean roots by CO + $NaHCO_3$ during this same period. Light reversed much of the inhibition of corn root respiration.

The partial reversal of the inhibition of bean root respiration by CO in the presence of bicarbonate ions could have resulted from bicarbonate ions affecting the same enzyme system as CO.

In several unreported tests inhibition by bicarbonate during the seventh and eighth hours was more than 50 %. In some of these tests the roots from half of the flasks were removed after the sixth hour, rinsed in distilled water and placed in Hoagland's solution. The inhibition was not reversed, although the roots were washed clean and placed in a low bicarbonate nutrient solution. This is evidence against the possibility that the inhibition of respiration in the bicarbonate solution resulted from the accumlation of bicarbonate ions around the root surfaces.

The larger percentage inhibitions during the first hour of these experiments than were reported in table I are in part a result of the longer time required for changing gases in the flasks before starting the measurements. Actually the first hour in table IV corresponds in time more closely with the second hour in table I. Differences in oxygen tension may also contribute to variations in ion effects.

It has been noted that the dry weights of bean roots placed in Hoagland's solution were always greater at the end of the experiments, without exception, than the dry weights of roots placed in a bicarbonate + Hoagland's solution, the differences in final weight were usually about 1 mg in 10 mg. This difference in weight was evident only with beans and may account for the activation of respiration found in the first hour with these roots. Possibly the roots in bicarbonate-nutrient solution absorbed less nutrients than those in the basal solution. In any event any weight changes during the course of an experiment would be reflected in an increase or decrease in the calculated rate of respiration.

DISCUSSION

The data reported in this paper indicate bicarbonate ions have an inhibitory effect on the respiration of several plant species, whereas the respiration of other species is but little affected by it. The plants that were affected are chlorosis susceptible. Plants resistant to chlorosis are only slightly affected by bicarbonate.

If bicarbonate ions affect the cytochrome oxidase system, differences in susceptibility to chlorosis would be explained. The apparent effect of bicarbonate ions on the cytochrome oxidase system is, however, difficult to explain. It is possible that absorbed bicarbonate ions would affect respiration at the substrate level. Insomuch as the cytoplasm of most cells is strongly buffered between pH 5.5 to 6.5, CO_2 liberated in decarboxylation reactions must undoubtedly exist within the cell as either gaseous molecules or as bicarbonate ions. Any external supply of bicarbonate, then would be expected to affect the concentration of bicarbonate ions or CO_2 and hence drive the equilibrium in the direction opposite decarboxylation. An increase of citric and malic acid could be accounted for in this manner. However, this does not exlpain why plants sensitive to copper deficiency and apparently being able to get along with copper terminal oxidases should not be affected in a similar manner at the substrate level.

The evidence obtained that CO and HCO_3^- inhibitions of bean root respiration are not additive and that HCO_3^- prevents in part the light reversal of CO inhibition supports the general thesis that the HCO_3^- ion may affect the cytochrome oxidase system.

Further inhibitory studies using sodium azide, sodium cyanide, and sodium diethyl dithiocarbamate have failed to shed additional light on the bicarbonate effect. It is highly probable that the $NaHCO_3$ effect on chlorosis-susceptible plants is more complicated than a simple inhibitory effect on the iron terminal oxidase.

SUMMARY

The effect of bicarbonate ions on oxygen uptake by excised roots of several chlorosis-susceptible and chlorosis-resistant plant species was determined on apical centimeter root sections in buffered nutrient solutions using the Warburg "direct method."

Apical sections of Great Northern bean roots took up oxygen at nearly uniform rates when immersed in nutrient solutions varied over the pH range from 4.5

to 8.5. At pH values of 3 and 10.5 there were marked reductions in the rate of respiration. Roots left 16 hours in a nutrient solution buffered at pH 9.4 showed only a slight decrease in rate of oxygen uptake.

The respiration of excised bean roots immersed in Hoagland's solution was not affected appreciably by Cl^-, $SO_4^=$, Ca++, and Na+ ions at concentrations of 25 meq/l. Bicarbonate ions inhibited the respiration markedly. The inhibition increased with time.

The rate of oxygen consumption by excised roots from a group of plants susceptible to lime-induced chlorosis was markedly reduced by bicarbonate ions. Roots from plants resistant to lime-induced chlorosis were only slightly affected by bicarbonate.

The inhibition of oxygen uptake by carbon monoxide with roots in standard Hoagland's solution was largely reversed by light, but in the presence of bicarbonate ions the carbon monoxide inhibition was only partially reversed by light.

Bicarbonate inhibition of respiration of excised roots is not fully explained by a direct inhibition of the cytochrome system.

The authors are indebted to F. B. Wann (deceased), L. L. Jansen, and H. B. Peterson for many helpful suggestions and criticisms during the course of this investigation.

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ABSORPTION OF IONIC SPECIES OF ORTHOPHOSPHATE BY BARLEY ROOTS: EFFECTS OF 2,4-DINITROPHENOL AND OXYGEN TENSION [1,2]

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The linkage between active salt absorption by plant roots and oxygen tension has been described by Hoagland and Broyer (6) and Steward et al (15). Despite these contributions, more detailed experiments employing shorter absorption periods and oxygen partial pressures below one percent are desirable. Information is needed with respect to orthophosphate, in particular, since early workers studied the absorption of bromide, nitrate, and potassium ions, as influenced by anaerobiosis.

From experiments described in this paper, it is found that each of the two first order reactions through which $H_2PO_4^-$ and $HPO_4^=$ ion species are absorbed (5), have an oxygen requirement. The overall rate of phosphate sorption is shown to be independent of the partial pressure of oxygen over the range from about 3 to 100 % oxygen, where the total pressure is one atmosphere. Another finding is that each of the two first order reactions is inhibited competitively by 2,4-dinitrophenol.

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Materials and Methods

Seed of barley, *Hordeum vulgare,* var. Atlas 46, were obtained from the 1954 crop grown in the vicinity of Davis, California. Twenty grams of seed were soaked for 24 hours in approximately one liter of demineralized water, aerated at a flow-rate of 1000 ml per hour at 24° C in a dark chamber. The sprouted seeds were then rinsed in demineralized water and distributed on boiled cheesecloth supported on a stainless steel screen and covered as described previously (5). Seedling roots were grown five days at 24° C in darkness in 1×10^{-4} M $CaSO_4$ solutions, aerated at a flow-rate of 2500 ml per hour per liter of solution, using glass tubing aerators with an inside diameter of 3 mm. Healthy roots 10 to 15 cm in length were obtained with this procedure. Roots were excised with clean

shears about 5 mm below the seeds. Excised roots were rinsed and placed in about 3 liters of demineralized water and thoroughly mixed. Experiments were performed using one gram of roots in phosphate solutions in equilibrium with nitrogen : oxygen gas mixtures containing oxygen at partial pressures ranging from 0.002 to 21 % oxygen.[3] Argon gas was not a contaminant in any of these mixtures.

Most of the dissolved oxygen was removed initially from demineralized water by boiling; then the gas mixtures were bubbled through either one liter volumes contained in Erlenmeyer flasks having standard taper joints, or 70-ml test tubes also fitted with standard taper joints. The gases escaped from the containers via exit tubes under the water surface in the water baths. On the basis of information received from workers at the Bureau of Standards, it was learned that where the flow rate was 2500 ml per hour, a one liter volume would reach equilibrium with the influent gas after 15 hours. Where the flow-rate was four-fold higher, a one liter volume would equilibrate after 3 hours, provided stoppers were employed as well as other precautions. In most cases, a flow-rate of 2500 ml per hour was found most convenient for use.

On leaving the steel cylinders, the gas mixtures passed through a scrubbing tower containing water, then through a cotton filter and into a copper or glass manifold. From this point the gas mixture flowed into the phosphate solutions. No more than three thick walled rubber tubing connectors were used in any gas line assembly. All joints in the copper manifold were carefully soldered, and no leaks could be detected in the assembly under the positive pressure head of about one pound used in these experiments.

Absorption experiments were usually done using one liter volumes in order to maintain the phosphate concentration and the pH essentially unchanged during one hour absorption periods. However, experiments in which solutions containing phosphate at 1×10^{-4} M and pH 4 were invariably done in 50 ml volumes, since changes in concentration were negligible and pH control was easily maintained during one-hour periods. In experiments with 2,4-dinitrophenol, experimental design was similar. DNP, obtained from Eastman Kodak Company, was made up in two stock solutions containing 18.4 and 184 μg DNP per ml respectively; from these, solutions were made ranging from 1×10^{-7} M to 1×10^{-3} M.

In preliminary experiments a system was employed where the phosphate solutions and the roots were emptied independently into the vessels following a period of time during which the entire assembly came to equilibrium with any particular gas mixture. The assembly was fitted with standard taper joints and adapters such that side-arm flasks could be swung independently through an arc of 180 degrees to permit emptying the contents of the side arm flasks into the main vessel of one liter volume. Through the use of this set-up, it was found that prior equilibration of roots with the liquid phase in equilibrium with low oxygen tensions was not critical. The critical point was the preparation and maintenance of solutions essentially in equilibrium with a given gas mixture. Choice of flow-rates was important in this respect.

The pH of solutions was adjusted to the desired value with 0.1 *N* KOH or HCl. The one-liter Erlenmeyer flasks or 70-ml test tubes containing the various solutions were placed in a water bath at 30° C and the solutions, under constant agitation of the inflowing gas mixture, came to the temperature of the water bath. Under aerobic conditions, the rate of phosphate absorption remained constant over the flow-rate range from 2,000 to 30,000 ml per liter × hour.

In all experiments the specific activity of the solutions used in absorption studies was 3.2 mc per gram P. A radioactive phosphate stock solution was made up beforehand to contain 40,000 mc per gram P, as described previously (5). At a given oxygen pressure or DNP concentration, phosphate was varied over the range from 10^{-4} M to 10^{-6} M, with the pH adjusted to 4 or 7.

Excised roots were removed from demineralized water in which they had been suspended following excision, blotted on cheesecloth, and one-gram portions weighed out on a torsion balance. At zero time, the roots were transferred to solutions containing the desired amount of tracer phosphate, provided sufficient time had elapsed for full equilibration of the liquid phase with the gas mixture. In all experiments, the maximum length of the absorption period was 60 minutes, unless otherwise indicated. At the end of absorption periods, the roots were rinsed and then transferred to solutions containing inactive phosphate and exchanged for 60 minutes, following which the roots were rinsed and dried under an infra-red lamp. Activity in the dry root samples was measured in a proportional counter using 90 % argon and 10 % methane gas purge. From preliminary experiments it was found that phosphate absorbed under anoxic conditions was bound in essentially an irreversible manner.

Design of Experiments: Ion absorption has been shown to be consistent with the following generalized scheme (3),

(*1*) The ion (M) combines with a metabolically produced carrier (R) to form a reversibly dissociable complex (MR); i.e., $M + R \rightleftharpoons MR$.

(*2*) The intermediate complex (MR) then breaks down, resulting in the liberation of the ion (M), and the regeneration of the carrier (R):

$$MR \rightleftharpoons M_{inside} + R_{regenerated}$$

From the expressions in (*1*) and (*2*), an equation can be derived which is formally analogous to that derived for enzyme kinetics by Michaelis and Menten (12). Lineweaver and Burk (9) later showed that on plotting the reciprocal, 1/v on the ordinate against the reciprocal 1/S on the abscissa, the results fall on a straight line as expressed by the linear function in equation (*3*).

[3] The amount of oxygen in each gas mixture was verified by the Gas Chemistry Section, Bureau of Standards.

$$\frac{1}{v} = \frac{K_m}{V_{max}} \cdot \frac{1}{S} + \frac{1}{V_{max}} \quad (3)$$

where (v) denotes the moles phosphate absorbed in one hour by one gram of excised barley roots; (S) denotes total phosphate concentration, hereafter expressed [ΣP]; V_{max} is the maximum absorption at infinite phosphate concentration, and K_m corresponds to the equilibria between phosphate and its carrier, or the apparent dissociation constant of the carrier-phosphate complex, under conditions where interfering ions are absent (5). In recent work, it was found that phosphate absorption could not be interpreted as consisting of a single first order reaction (5). These findings are confirmed in this work.

A method was developed by Hofstee (7), for determining whether more than one independent reaction is acting on a single substrate, or on different substrates which are in a non-rate limiting equilibrium. This method has been applied in the present work. By plotting the observed absorption v, against v/[ΣP], one obtains a curving line like that for the control line

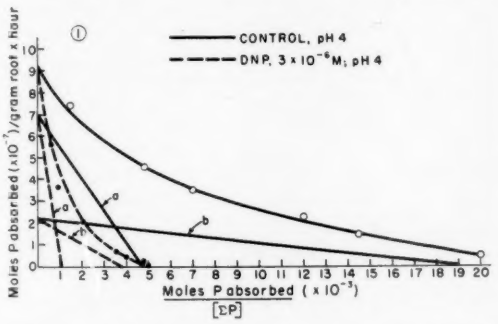

FIG. 1. Graphical separation of reactions involved in phosphate absorption at pH 4, in presence and absence of 2,4-dinitrophenol.

in figure 1. The curve may be resolved into linear components by use of polar coordinates, but in the case where the dissociation constants, K_m, differ widely from one another, the function can be solved by extrapolation as described previously (5). Intercepts with the ordinate for the two first order reactions, a and b, give V_{max} directly, and the values of the slopes give apparent dissociation constants, K_m, in respect to total phosphate concentration, [ΣP]. Since K_m expresses the equilibria between specific ion species and respective carriers, the constants have to be calculated in terms of the concentration of each ion species, $H_2PO_4^-$ and $HPO_4^=$. Knowing already the relative concentration of each ion species at a given pH value, one recalculates the slope of each line, a and b in figure 1, on the basis of the total concentration of each ion species, by use of the method of least squares (14). In order to determine the manner in which molecular oxygen and the inhibitor 2,4-dinitrophenol enter phosphate absorption reactions, it is necessary to extend the kinetic analysis discussed above. A critical discussion of three types of inhibitions can be found in the paper by Epstein and Hagen (3). Other types of inhibitions are known and have been described (4). A convenient method for distinguishing competitive from non-competitive inhibition is afforded by determining if the slope of the line increases in the reciprocal plot 1/v versus $1/[H_2PO_4^-]$, or $1/[HPO_4^=]$, while the intercept remains constant, as for example in figure 2. In non-competitive inhibition, the ratio between reciprocal slope and intercept remains essentially constant, while in competitive inhibition, the ratio increases, tables I and II, columns 2 and 3.

EXPERIMENTAL RESULTS

An important feature of experiments dealing with agents which can alter reaction rates is the unequivocal demonstration of reversibility in the system under study. With respect to phosphate absorption by barley roots, it can be shown that the inhibited reaction can return to an absorption rate typical of uninhibited roots. Results in figures 3 and 4 were obtained from experiments employing a range of concentrations of molecular oxygen, or 2,4-dinitrophenol. Roots were placed in solutions in equilibrium with various oxygen partial pressures, with phosphate at 1×10^{-4} M, pH 4, for intervals of 60 to 75 minutes, curves a and b, figure 3. Following these time intervals, air was introduced into the solutions and absorption went on under aerobic conditions for an additional 90 minutes. Results from similar experiments, employing oxygen tensions of 0.3 % and 21 % oxygen are also shown in figure 3, curves c and d. Apparently, the absorption mechanism is altered in an irreversible manner following 75 minutes exposure in solutions in equilibrium with an oxygen tension of 0.05 %, curve a. Following a 60-minute exposure to 0.05 % and 0.3 % oxygen, curves b and c, a steady rate is attained equal to that for fully aerobic roots, curve d. The initial absorption "lag" observed in the first leg of curves a, b, and c, was found to occur equally at pH 7 as at pH 4. The possibility that the "lag" was due to a slow rate of saturation of the external solution with air, or to excretions of unknown compounds from anoxic roots, was ruled out by replacing solutions present at the end of the anaerobic periods with fresh solutions in equilibrium with air. No change occurred in the character of the aerobic recovery curves over a 90-minute period.

Results in figure 4 were obtained with varying DNP concentrations. Following a 60-minute absorption period in "aerobic" solutions containing known amounts of DNP, and phosphate at 1×10^{-4} M, the roots were rinsed and placed in "aerobic" solutions containing phosphate 1×10^{-4} M, pH 4, for 90 minutes. The absorption reactions are reversible following DNP treatments at and below concentrations of 3×10^{-6} M, curves c and d, figure 4. The absorption rate following 60 minutes in DNP, 1×10^{-5} M, pH 4, was one-third the normal rate, curves a and e. Phosphate absorption is approximately half-maximal at a DNP concentration of 3×10^{-6} M in solutions containing phosphate 1×10^{-4} M, pH 4.

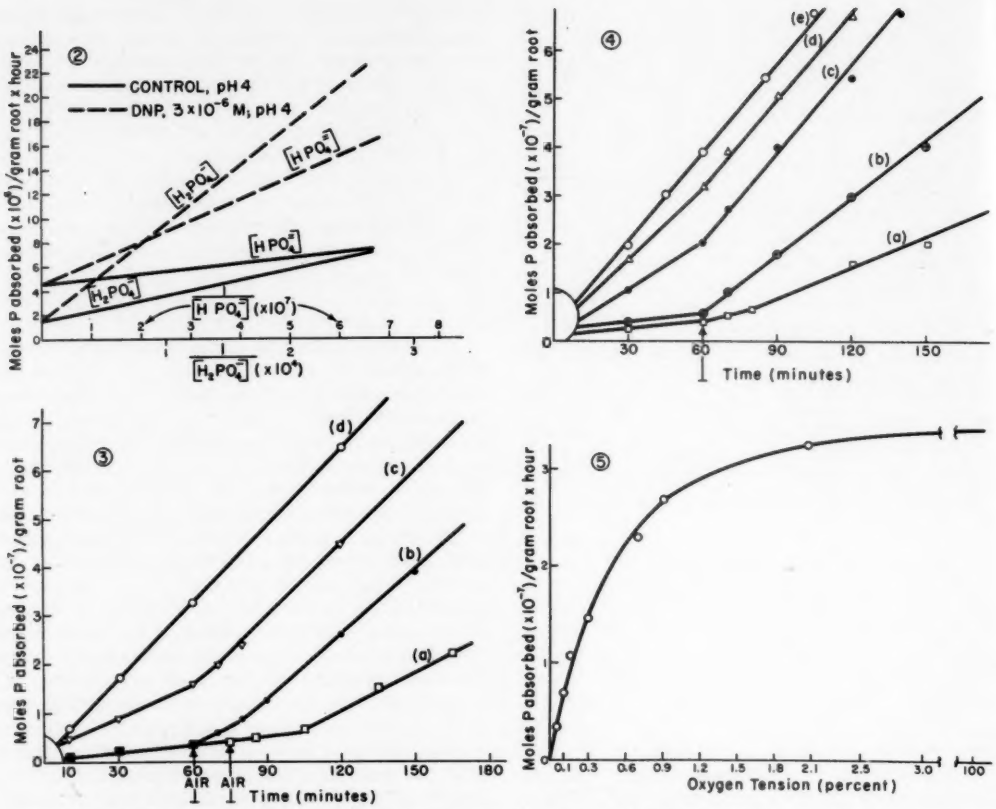

FIG. 2. Calculated curves showing competitive inhibition of the absorption of ionic species of orthophosphate by DNP 3×10^{-6} M, pH 4.

FIG. 3. Reversibility of phosphate absorption reactions following anoxic exposure; in curve a, arrow indicates introduction of air after 75 min exposure to 0.05 % oxygen; in curves b and c, air introduced after 60 min exposure to 0.05 % and 0.3 % oxygen, respectively; curve d, phosphate absorption in air. In each case, phosphate concentration was 1×10^{-4} M, and pH 4.

FIG. 4. Reversibility of phosphate absorption reactions following 60 min exposure to various concentrations of 2,4-dinitrophenol. Arrow indicates point at which roots were rinsed and returned to phosphate solutions 1×10^{-4} M, pH 4. Curve a, DNP 1×10^{-5} M; curve b, DNP 5×10^{-6} M; curve c, DNP 3×10^{-6} M; curve d, DNP 2×10^{-6} M; curve e, control.

FIG. 5. Relation between oxygen tension and phosphate absorption by excised barley roots in phosphate solutions 1×10^{-4} M, pH 4.

Between the limits from 5 to 60 minutes, absorption rate is linear and reversible at all oxygen tensions from 0.05 % and above, and all DNP concentrations at and below 3×10^{-6} M. Barley roots used in these experiments become flaccid after 25 minutes exposure to 0.002 % oxygen in solutions containing phosphate 1×10^{-4} M, pH 4. Similarly, 2 to 3 hours exposure to 0.05 % oxygen or DNP concentrations greater than 5×10^{-6} M causes the roots to become flaccid and defunct (unpublished data).

Absorption rate is independent of oxygen partial pressure over the range from about 3 to 100 % oxygen, figure 5. A hyperbolic relationship exists between the phosphate absorption rate and oxygen tension between the limits of 0 and 3 %. At an oxygen tension of 0.30 %, phosphate sorption rate is half-maximal, and maximal at 3.0 %, in solutions 1×10^{-4} M with respect to phosphate and at pH 4.

FURTHER EXPERIMENTS WITH 2,4-DINITROPHENOL: The effect of DNP on phosphate absorption was studied using phosphate concentrations over the range from 10^{-6} M to 10^{-4} M, at pH 4 or pH 7, and at one or more DNP concentrations. When the observed absorption during one hour, v, is plotted against the variable $v/[\Sigma P]$, one obtains the curve shown in figure 1. The curve may be resolved into its linear components as described previously under Methods. Intercepts with the ordinate for the two first order reactions a and b, give V_{max} directly for each reaction, and the values of the slopes give ap-

parent dissociation constants, K_m, in respect to total phosphate concentration [ΣP]. As previously explained, when one recalculates the slope of each line on the basis of the total concentration of the specific ion species, it is possible to re-plot the function in terms of a conventional Lineweaver and Burk plot, as shown in figure 2. Inspection of these reciprocal plots shows that DNP competitively inhibits the absorption of both $H_2PO_4^-$ and $HPO_4^=$ ions, thus indicating that DNP is bound at the same reactive group on the carrier as the phosphate ion species.

Quantitative expression is given to the manner in which DNP enters phosphate absorption reactions in table I, columns 2, 3, 6, and 7, in terms of numerical values for reciprocal intercept and slope. The apparent dissociation constants corresponding to the equilibria between $[H_2PO_4^-]$ and $[HPO_4^=]$ and their respective carriers $[R_a]^+$ and $[R_b]^+$ for systems without the inhibitor are given in columns 4 and 7. Similar values corresponding to the equilibria for systems containing DNP are given in columns 5 and 9. In the presence of DNP, 3×10^{-6} M, pH 4, the slope increases and the intercept remains unchanged from that of the untreated system. Results in figure 2 show this in a graphic manner, and indicate further that the inhibitory effect of DNP is abolished by increasing concentrations of the specific ion species. Apparently, the affinity of DNP for each of the intermediate complexes is of the same order of magnitude, table I, columns 5 and 9. The concentration of DNP producing half-maximal inhibition is 3×10^{-6} M, in solutions 1×10^{-4} M with respect to phosphate, pH 4. At pH 7, half-maximal inhibition occurs at a DNP concentration of 1×10^{-4} M, representing a thirty-fold higher concentration of DNP (unpublished data).

FURTHER EXPERIMENTS WITH MOLECULAR OXYGEN: In this study, molecular oxygen is considered an inhibitor, though in a sense which is the reverse of that usually applied. For instance, phosphate sorption by barley roots is a hyperbolic function of the degree of saturation of an unspecified site with molecular oxygen, between the limits of oxygen partial pressures from about 3 to 0 %, figure 5. As in the experiments with DNP, quantitative expression is given to the manner in which molecular oxygen enters phosphate absorption reactions, by the extent of change in the reciprocal intercept and slope when oxygen becomes limiting, table II. Both intercept and slope change each time oxygen tension changes, and by approximately the same amount as shown in columns 2, 3, 6, and 7. In a simple system, this would be analogous to a non-competitive inhibition and would indicate that molecular oxygen combines with both the carrier and the carrier-phosphate intermediate complex at a site or reactive group different from that occupied by phosphate ions. This behavior implies that the response of the phosphate absorption reactions to oxygen stress is independent of the phosphate concentration in the external medium. This was indeed found to be the case over the range of phosphate concentrations from 2×10^{-6} to 4×10^{-4} M, at pH 4 or 7. Caution should be exercised, however, in broadly generalizing from application of kinetic analysis, developed originally for simple systems, to complex systems like the phosphate absorption mechanism. At the cellular level, for instance, one is usually working with specific binding sites separated spatially within a reaction sequence.

Results shown in table II, columns 5 and 9, indicate that the affinity of molecular oxygen is of the same order of magnitude for each of the carriers $[R_a]^+$ and $[R_b]^+$. The maximum absorption of the ionic species of orthophosphate at infinite phosphate concentration, V_{max}, is shown in table III. Absorption of $H_2PO_4^-$ is lowered to a slightly greater extent than $HPO_4^=$ at all oxygen tensions below 21 %.

TABLE I

COMPETITIVE INHIBITION OF ABSORPTION OF IONIC SPECIES OF ORTHOPHOSPHATE BY 2,4-DINITROPHENOL, pH 4

	[2,4-DINITROPHENOL]	
	NONE	3×10^{-6} M
($R_aH_2PO_4$)		
Intercept, $\frac{\text{Moles}^{-1}}{\text{M/L}^{-1}}$ ($\times 10^6$)	1.42	1.48
Slope, Moles^{-1} ($\times 10^2$)	2.07	7.86
K_m, Moles ($\times 10^{-4}$)	1.46	...
K_i, Moles ($\times 10^{-6}$)	...	1.14
(R_bHPO_4)		
Intercept, $\frac{\text{Moles}^{-1}}{\text{M/L}^{-1}}$ ($\times 10^6$)	4.50	4.50
Slope, Moles^{-1} ($\times 10^{-2}$)	3.80	16.63
K_m, Moles ($\times 10^{-6}$)	8.44	...
K_i, Moles ($\times 10^{-6}$)	...	1.01

TABLE II

"NON-COMPETITIVE" NATURE OF THE OXYGEN EFFECT ON ABSORPTION OF IONIC SPECIES OF ORTHOPHOSPHATE, pH 4

	OXYGEN PARTIAL PRESSURE		
	21 %	0.71 %	0.30 %
($R_aH_2PO_4$)			
Intercept, $\frac{\text{Moles}^{-1}}{\text{M/L}^{-1}}$ ($\times 10^6$) ..	2.08	3.53	5.44
Slope, Moles^{-1} ($\times 10^2$)	1.25	2.27	2.78
K_m, Moles ($\times 10^{-5}$)	6.00	...	...
K_i, Moles ($\times 10^{-5}$)	...	2.60	1.20
(R_bHPO_4)			
Intercept, $\frac{\text{Moles}^{-1}}{\text{M/L}^{-1}}$ ($\times 10^6$) ..	19.60	27.40	28.90
Slope, Moles^{-1} ($\times 10^{-2}$)	3.90	7.20	8.00
K_m, Moles ($\times 10^{-9}$)	2.00	...	...
K_i, Moles ($\times 10^{-5}$)	...	6.50	3.80

DISCUSSION

Certain assumptions shown to be valid from previous work are applied implicitly in this study (5).

TABLE III

MAXIMUM ABSORPTION OF IONIC SPECIES OF ORTHOPHOSPHATE, V_{max}, IN RELATION TO OXYGEN TENSION, pH 4

Oxygen pressure, %	$V_{max}[R_aH_2PO_4]$ Moles ($\times 10^{-8}$)	Percent of control	$V_{max}[R_bHPO_4]$ Moles ($\times 10^{-8}$)	Percent of control
21.0	48.1×10^{-8}	100	5.1×10^{-8}	100
0.71	28.3×10^{-8}	59	3.6×10^{-8}	71
0.30	18.4×10^{-8}	38	3.5×10^{-8}	69
0.05	6.0×10^{-8}	12	1.3×10^{-8}	25

Briefly, these assumptions are that, 1) one mole of phosphate ion species may combine with the carrier; 2) the rate of phosphate absorption is proportional to the concentration of the carrier-phosphate complex; the uptake rate reaching a maximum value when the carrier is saturated with phosphate; and 3) the rate-limiting step is the breakdown of the intermediate complex.

In this study, the kinetic analysis is made under conditions where the results from inhibitor experiments accurately measure the rate-limiting step. Furthermore, it is desirable to point out that barley roots are favorable material for use in studies relating oxygen tension to a process such as phosphate sorption. In the first place, the radius of the barley roots used in this work is approximately 0.02 cm. Calculations reveal that the rate of oxygen diffusion into root cells cannot be a limiting factor in these experiments.

2,4-Dinitrophenol competitively inhibits the absorption of both $H_2PO_4^-$ and $HPO_4^=$ ions under conditions where hydroxyl ions do not compete, namely; pH 4. This finding may offer a clue to the isolation of the carrier-phosphate complexes in barley roots. Dinitrophenol has long been known to produce an uncoupling of phosphorylations from respiration (10). Speculation concerning the significance of the competitive inhibition of phosphate absorption by DNP is unprofitable, since the site at which DNP acts has not been determined (1). Furthermore, the relationship between oxidative phosphorylations via the respiratory chain, and phosphate sorption by barley roots remains to be established.

Results from experiments where pH is varied between 4 and 7, show that the penetration of membranes by DNP is facilitated at pH 4. This finding is in agreement with the work of Beevers (2), and Newcomb (13), both of whom studied the effect of DNP on respiration. Presumably, penetration occurs as the undissociated molecule, following which DNP ionizes and combines with each of the two carriers with equal affinity.

At 30° C phosphate absorption is half-maximal at an oxygen partial pressure of about 0.3 %, figure 5. This value for phosphate absorption in relation to oxygen tension is much lower than that previously reported for bromide and potassium ions (6). According to Ludwig and Kuby (11), half-maximal oxidation of cytochromes a_3, a, and c, occurs in yeast cells at an oxygen tension of about 0.2 % at a temperature of 25° C. This correlation is submitted as suggestive evidence that cytochrome oxidase is the terminal oxidase in the six-day-old barley roots used in this study. Temperature has a profound influence on the affinity between cytochrome oxidase and molecular oxygen (8).

Information on the equivalence, in moles, between molecular oxygen and the carriers $[R_a]^+$ and $[R_b]^+$ is obtained by application of equation (4) to the results of figure 5.

$$(4) \qquad \frac{v}{v_i} = 1 - \frac{(I)^r}{K_i}; \ (16),$$

where (v) is absorption rate in air, (v_i) is absorption rate at some oxygen tension lower than 3 %; (I) is the oxygen tension, and (r) is the number of molecules of oxygen combining with one molecule of the carrier. The value for (r) is determined by estimating the slope of the line obtained when log $((v/v_i) - 1)$ is plotted against log (I). In the overall reaction, it is found that 1.1 moles of oxygen combine with both carriers. When the overall reaction is broken down into each of the two first order reactions, one finds that approximately 20 % less oxygen is required for the reaction involving $HPO_4^=$ than that for the $H_2PO_4^-$ ion.

SUMMARY

The effect of oxygen tension and 2,4-dinitrophenol on phosphate absorption by excised roots of *Hordeum vulgare*, variety Atlas 46, was studied. Quantitative expression is given to the manner in which molecular oxygen and dinitrophenol enter phosphate absorption reactions.

1. Dinitrophenol competitively inhibits the absorption of both $H_2PO_4^-$ and $HPO_4^=$ ions.

2. Phosphate sorption is independent of oxygen tension over the range from about 3 to 100 %, where the total pressure is one atmosphere.

3. At an oxygen tension of 0.3 %, phosphate absorption rate is half-maximal, and over the range from 3 to 0 % oxygen tension, the relation is typically a hyperbolic function.

4. At an oxygen tension of 0.71 %, the maximum rate of phosphate absorption at infinite phosphate concentration for the first order reaction involving $[H_2PO_4^-]$ was lowered 41 % and that for $[HPO_4^=]$ was lowered 29 %. Hence, both first order reactions have an oxygen requirement.

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BRIEF PAPERS

FURTHER STUDIES OF SOME PLANT INDOLES AND AUXINS BY PAPER CHROMATOGRAPHY[1,2]

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The characterization of plant growth substances by paper chromatography has expanded rapidly in the past few years, and since the review of Gordon in 1954 (6) many new papers have appeared (1, 2, 4, 7, 11, 12, 17, 19, 20, 21, 22, 24, 26). This increasing use of the procedure makes it desirable to report both its successes and limitations when applied to new plant materials and to the solution of existing problems.

Since all the auxins so far conclusively identified in plants are indole derivatives, it was felt that an examination of plant material known to be rich in indole compounds might bring out some new auxin relationships. Chromatograms of two indole-rich materials have accordingly been prepared and studied. The indole derivatives of a representative tissue culture and of the medium on which it was grown have also been examined, in order to determine whether the indoleacetic acid in the culture could have been taken up from the medium.

Indole itself has been reported to be present in the perfume of, and extracts from, many flowers, notably by Hesse (8, 9), who succeeded in isolating the substance in from 0.1 to 2.5 % yield from large quantities of Citrus and other flower oils. Since no modern investigation of this material has been recorded, a study was made of orange blossoms obtained through the courtesy of Dr. W. C. Cooper, U. S. Department of Agriculture, Weslaco, Texas. The blossoms were picked and shipped by air mail the same day. The material was wilted and somewhat browned on arrival, but was at once placed in the deep freeze at – 10° C. Later, the 139 gm of entire flowers were extracted with 800 ml of peroxide-free ether for 2 hours at 5° C. The strongly yellow ether extract was fractionated into neutral and acid substances by extraction of the acid materials with 1 % $NaHCO_3$, separation of the aqueous layer, acidification to pH 3 with 15 % tartaric acid, and re-extraction with ether. Both fractions were strongly fragrant; on evaporation the acid fraction yielded about 0.4 ml of a yellow oil, while the neutral fraction produced a copious waxy orange-colored residue. This latter was triturated with several small volumes of ethanol, which were combined and evaporated in vacuo, resulting in an orange oil. Samples of this alcohol triturate oil, the acid fraction oil and a small piece of the neutral fraction wax were applied to individual Whatman No. 1 paper strips and chromatographed in 8–1–1, isopropanol–28 % NH_4OH–H_2O. The appearance of the strips under ultraviolet light and after spraying with modified Salkowski reagent is indicated in table I.

Methyl anthranilate, also reported by Hesse as a constituent of orange blossom oil, was run on the control strips where it appeared as a bright blue fluorescing spot, but no similarly appearing compound was noted on chromatograms of the extracts. The presence of indole is confirmed by the orange Salkow-

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TABLE I

CHROMATOGRAPHIC BEHAVIOR OF SALKOWSKI-POSITIVE SUBSTANCES IN FRACTIONS OF ETHER EXTRACT OF CITRUS FLOWERS

COMPOUND	CONTROLS		ACID FRACTION		NEUTRAL WAXY FRACTION		NEUTRAL ALCOHOL TRITURATE	
	SALKOWSKI COLOR	R_f	SALKOWSKI COLOR	R_f	SALKOWSKI COLOR	R_f	SALKOWSKI COLOR	R_f
Indole, 40 μgm	Diffuse orange	0.8	Weak orange	0.8–0.9	Diffuse orange	0.7–0.9	Orange	0.7–0.9
Methyl anthranilate, 20 μgm	Blue (in UV)	0.77	Faint brown	0.53			Yellowing	0.4–0.7
IAA, 20 μgm	Crimson	0.25	Crimson	0.20–0.25	Bright yellow (in UV)	0.2–0.4	Bright yellow (in UV)	0.2–0.35
			Yellow (in UV)	0.12	Bright yellow (in UV)	0.0–0.1	Bright yellow (in UV)	0.04–0.1
					Green	0.0		

Equilibrated 18 hrs, run 10 hrs at 26° C, solvent: isopropanol–water–ammonia, 8–1–1.

ski reaction at high R_f's on all three strips; apparently so much was present that it was carried over into the acid fraction as well. As has been noted elsewhere (24) indole does not form compact spots under these chromatographic conditions. Two brilliant yellow fluorescing compounds are present in the neutral extracts. Indole acetic acid (IAA), probably extracted from the ovaries of the flowers, was indicated by a compact crimson area at R_f 0.20 to 0.25.

The acid fraction was further studied by biological assay. Portions of the chromatogram were placed in 2 ml of 2.5 % sucrose solution together with twelve 3-mm subapical Avena coleoptile sections and the growth over 20 hours was measured. The detection of an area of strong growth promotion at an average R_f of 0.25, corresponding with a control spot of synthetic IAA, supported the above colorimetric identification of IAA in the orange blossom extract. IAA has previously been reported to occur in flower and fruit parts (18, 20). In addition to the area of growth promotion considered due to IAA, an area of growth inhibition was detected on the above chromatogram. The latter area corresponded in R_f with the unknown substance present in a number of tissues and termed inhibitor β (12). Kefford (13) has recently found evidence that inhibitor β may be an indole or a phenol derivative.

Another indole derivative, abrine (n-methyltryptophan) constitutes about 1 % of the dry weight of Jequirity seeds, *Abrus precatorius* L. (5). Therefore, 400 gm of these seeds obtained from the Cheney Drug Company, Boston, Massachusetts were subjected to the isolation procedures for abrine described by Cahill and Jackson (3). No difficulty was experienced in obtaining it as a pure crystalline compound. Nevertheless, several chromatograms of the same methanol extract from which abrine was later isolated pure failed to show any convincing reaction with either the Salkowski or Ehrlich reagents. The isolated abrine, when chromatographed alone, was readily detected on the paper by both these reagents at an R_f of 0.29 (cf 24). Evidently, therefore, the large quantity of pigments removed from the seed coat, as well as the lipids present in the extract, interfered with the chromatography and color-developing reagents. The experience is cited to demonstrate how easily compounds present in considerable quantities in an extract may be completely masked by other substances.

The isolation of a strain of corn endosperm which grows vigorously in vitro has been reported by La Rue (16), and its cultural requirements have been described by Straus and La Rue (25). The tissue is designated as CE Clone 1-C and has been maintained for several years on a mineral salts–sucrose–yeast extract medium.

We obtained from Dr. Jacob Straus of these laboratories (cf 25) a group of 16 cultures of this endosperm material of the same age. The combined fresh weight of the tissue was 19 gm, and being very friable, it was not ground but extracted directly with 200 ml of peroxide-free ether in the cold room overnight.

TABLE II

R_f AND SALKOWSKI REACTION OF ETHER EXTRACT (ACID FRACTION) FROM CORN ENDOSPERM CULTURED ON YEAST EXTRACT MEDIUM

MATERIAL	R_f	SALKOWSKI REACTION
IAN, 40 μgm	0.84	Blue
IAA, 40 μgm	0.30	Crimson
Acid fraction of ether soluble extract	0.30	Crimson
	0.20	Two close crimson spots
	0.17	

Equilibrated 6 hrs, run 15 hrs at 26° C, solvent: isopropanol–ammonia–water, 8–1–1.

Fractionation and chromatography of this extract was carried out as outlined above for the orange blossom material; in this case both the neutral and acid fractions of ether-soluble material yielded only very small quantities of residue. Table II presents the results for the acid fraction and controls only; the chromatograms of the neutral fraction showed no spots other than faint fluorescent areas.

From the observed color density and spot area, at least 20 μgm of IAA were present in this 19 gm of tissue, or approximately 1 mg/kg fresh weight. This concentration is of the same order as those found by Hinsvark et al (10) in developing corn kernels, but it is remarkably high when compared with other growing tissues (e.g., etiolated seedlings) in which IAA has been assayed. The two close but distinctly separated spots of lower R_f value than IAA had the same R_f and gave the same crimson color with the Salkowski reagent as two bands found in alcoholic extracts of sweet corn seed and potato tuber skin (13). These spots are in the region to which indole pyruvic acid migrates, and this compound gives a similar color reaction (24). However, since direct comparison with a new sample of synthetic material was not possible at this time, precise identification of these spots must be deferred.

The large amount of IAA (1 mg/kg) present in the maize endosperm must either have been synthesized by this tissue, or have been concentrated from the medium. The IAA content of the medium was therefore examined. The medium used to grow the 16 cultures totalled about 160 ml and included some 800 mg of Difco yeast extract. Accordingly, 2.5 gm of Difco yeast extract was dissolved in 100 ml of water. One half of this solution was then autoclaved 20 minutes at 15 lbs to see if this procedure would release IAA from labile precursors. (The yeast extract used for the tissue culture medium was Seitz-filtered and had not been autoclaved.) When cool both the autoclaved and untreated extracts were brought to pH 3 with 15 % tartaric acid and extracted twice with 100 ml of peroxide-free ether. A very small amount of tan-colored residue resulted from the evaporation of the ether. Chromatograms were run as described above. Although both chromatograms showed several small compact spots of fluorescent ma-

terials, no trace of any color was developed by the Salkowski reagent.

Since the total amount of substances which might interfere was very small it is unlikely that IAA actually present could have been masked as was the abrine in the Jequirity seeds extract. The modified Salkowski reagent could readily detect 1 μgm of IAA on clean strips such as these. It can be concluded, therefore, that less than 1 μgm of IAA is present in a gram of yeast extract, and that autoclaving at its natural pH does not release any detectable amount of IAA or other Salkowski-reacting compounds from yeast extract in solution.

However, yeast extract must contain some IAA or other ether-soluble auxin, since IAA has been isolated from yeast (14), and more recently Skinner and Street (23) found it necessary to treat yeast extract with ether to remove auxin-like substances interfering with their root cultures. To check this point, portions of a chromatogram of the ether-soluble acids from 2.5 gm of Difco yeast extract were assayed with Avena coleoptile sections. An area of growth activity corresponding in R_f value with IAA, and equivalent to about 0.4 μgm of IAA, was detected. This corresponds to about 0.15 μgm IAA/gm (Skinner and Street estimated 0.4 μgm/gm). Hence, if the endosperm cultures had taken up all the IAA from the 800 mg of yeast extract present in the medium, they would only have gained 0.13 μgm. But the IAA content of the tissues, as shown above, was certainly a hundred-fold more than this, namely of the order of 20 μgm. Thus, it is clear that the endosperm culture synthesizes its IAA de novo, and does not merely absorb it from the medium. This provides independent support of the conclusion of Kulescha and Gautheret (15) based on bioassay alone, that some tissue cultures are capable of producing an auxin de novo.

In this and related studies it has been observed that the Salkowski reaction is by no means specific for indoles. Many phenolic compounds give strong reactions. Some colors and R_f's are: catechol, brown-pink, 0.4; *p*-hydroxybenzaldehyde, faint yellow, 0.4; resorcinol, crimson, 0.7; α-naphthol, blue, 0.75; orcinol, yellow-green, 0.8. This fact should be noted in studying plant extracts.

Summary

Paper chromatography, using a modified Salkowski reagent and biological assay, was applied to a number of plant extracts believed to contain indole derivatives. Orange blossoms were shown to contain quantities of indole and indole-3-acetic acid (IAA) together with an unknown growth inhibitor.

Although crystalline abrine was readily obtained from a methanolic extract of Abrus seeds, no abrine could be detected on a chromatogram of this extract, because of interference by lipides and pigments.

Yeast extract (Difco) contains IAA at a concentration of about 0.15 μgm/gm.

Corn endosperm grown in vitro, using the yeast extract as medium, contains 1 mg IAA per kg fresh weight, as well as two other Salkowski reacting substances. These compounds are thus synthesized by the tissue and not merely taken up from the medium.

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AN AUXIN INACTIVATION SYSTEM INVOLVING TYROSINASE[1,2]

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Since the discovery by Thimann (12) that leaf extracts of *Vicia Faba* and *Helianthus annuus* would inactivate native auxins in solution, much work has been done to elucidate the nature of the auxin-inactivating systems in extracts of plants. Because auxin destruction tended to be pronounced in tissue extracts in which browning occurred, it was suggested that phenolases might participate in auxin destruction (12, 15). Wagenknecht and Burris (13) found inhibitions of IAA oxidation by copper-chelating agents, which further implicated a copper enzyme. These results were not widely confirmed, however, and more recently the emphasis has shifted to the iron enzyme, peroxidase. There is conclusive evidence that peroxidase can inactivate IAA, although the mechanism by which this oxidation comes about remains uncertain (4, 5, 6, 7, 9, 11).

In recent work (2) on extracts of the fern *Osmunda cinnamomea* L., peroxidase appeared to be responsible for at least part of the auxin-destroying activity found in tissue extracts. However, occasional inhibitions by copper-chelating agents were observed. Since these extracts also contained an active tyrosinase, it appeared worthwhile to investigate the possibility that this enzyme might act on IAA.

Tyrosinase, prepared from the mushroom *Psalliota campestris*, was obtained from the Nutritional Biochemicals Corporation, Cleveland, Ohio. Tyrosinase activity was determined by the method of Adams and Nelson (1). The tyrosinase-containing extract of Osmunda was prepared by extracting dormant leaves as described previously (2), and dialyzing the centrifuged extract against 0.067 M, pH 6.1 phosphate buffer for 5 days (with five changes of buffer), at 4° C. It contained 0.74 catecholase units per ml, or 2.1 units per gm fresh weight of tissue.

IAA inactivation was measured by incubating a 7×10^{-7} M solution of IAA, containing appropriate addenda and made up to 10 ml, at 30° C, in the dark, with occasional shaking. Aliquots were removed at the times specified, boiled for 5 minutes to inactivate the enzyme, and made into agar blocks for auxin bioassay by the standard Avena curvature test (14). Within the range of variability of the test, Avena curvature was roughly proportional to the concentration of auxin in the aliquot. Therefore, all results are expressed as degrees Avena curvature, and percentage inactivation is calculated from these values.

The results shown in table I indicate that, while mushroom tyrosinase alone or in the presence of tyrosine or 3,4-dihydroxyphenylalanine (DOPA) did not inactivate IAA, appreciable auxin destruction occurred when the enzyme acted on pyrogallol or catechol. These phenols alone had no effect on IAA, and all four of them were oxidized by the enzyme, as was

TABLE I

INACTIVATION OF IAA BY MUSHROOM TYROSINASE

ADDENDA *	TIME	AVENA CURVATURE	INACTIVATION
	hrs	*degrees*	*%*
None	6	33	..
Enzyme	0	32	
	6	35	0
Pyrogallol	6	34	0
Enzyme + pyrogallol	0	32	
	6	23	27
Catechol	6	31	6
Enzyme + catechol	0	32	
	6	14	58
DOPA	6	33	0
Enzyme + DOPA	0	33	
	6	32	4
Tyrosine	6	32	4
Enzyme + tyrosine	0	32	
	6	33	0

* Basal reaction mixture was 7×10^{-7} M IAA, pH 6.1. Tyrosinase was added to give 0.12 catecholase units per ml. Phenols were added to make 10^{-3} M. Final volume 10 ml.

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apparent from the formation of colored oxidation products in the solutions.

This experiment suggests that IAA reacts with products of enzymatic oxidation of catechol and pyrogallol. Certain alternative interpretations, however, need to be considered:

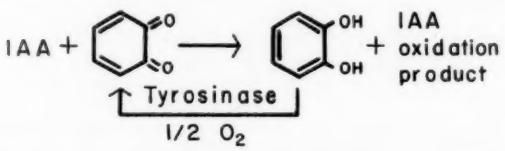
1) The oxidation of catechol or pyrogallol may give rise to an inhibitor of the Avena test. However, IAA added to the reaction mixture at 6 hours, after completion of catechol oxidation, was fully recovered in the bioassay, showing that catechol oxidation products do not, in fact, inhibit Avena curvature.

2) When the reaction mixtures were boiled at the end of six hours, the IAA might have reacted with the phenol oxidation products in a heat stimulated reaction, and might have been present in undiminished amount until that time. This suggestion was disproved by the fact that the full activity of IAA was recovered when it was added to the catechol-tyrosinase reaction mixture at six hours, and an aliquot then boiled and assayed.

3) The possibility that contamination by peroxidase or other IAA-oxidizing enzymes was responsible for auxin inactivation seems exceedingly unlikely, since pyrogallol and catechol are among the most powerful inhibitors of IAA oxidation by peroxidase and "IAA oxidase" (5, 6, 9). Furthermore, no peroxidase activity could be detected in the tyrosinase preparation, as determined by the technique of Sumner and Gjessing (10).

Manometric and spectroscopic measurements showed that the conversion of catechol to final oxidation products was complete at 6 hours, under the conditions shown in table I. When IAA was added to the system at this time, and incubation continued for another 6 hours before boiling and assay, full auxin activity was recovered. Therefore, the destruction of IAA observed in table I must have been brought about by the intermediate compounds formed during catechol oxidation (8), rather than final products.

At least two types of reactions between IAA and a quinonoid intermediate in polyphenol oxidation may be considered as a possible explanation of the observed inactivation. Firstly, IAA might reduce a quinone, leading to oxidation of IAA in a coupled system like that by which tyrosinase oxidizes ascorbic acid, TPN, and other substances:



Secondly, quinones might condense directly with the indole nucleus of IAA, as they are known to do with certain other indole compounds (3). Such reactions may, indeed, occur in the tyrosinase-catalyzed formation of melanin. The first possibility, coupled oxidation, should result in an increased oxygen uptake per mole of catechol, in the presence of IAA. Direct condensation might result in no effect on oxygen uptake, or an increase or decrease, depending on the nature of reactions subsequent to the condensation. Only a slight and doubtfully significant increase in total oxygen uptake with catechol was found when IAA was present at 4×10^{-3} M. The mechanism of auxin inactivation by the tyrosinase system is at present under investigation.

Experiments with the dialysed extract of Osmunda leaves indicated that this preparation would carry out the same type of auxin inactivation, when catechol was added, as did the mushroom tyrosinase. The results presented in table II show that in the presence of catechol and the extract, IAA disappears, while in the presence of the extract alone, almost all of the IAA is recovered. The additional inactivation in the presence of catechol may be presumed to be due to the tyrosinase which the extract contains.

The partial and variable inhibitions, by thiouracil and phenylthiourea, of IAA destruction in crude extracts of Osmunda leaves, observed previously (2), may now be given a tentative interpretation. The extent to which a copper-enzyme inhibitor would inhibit auxin inactivation in such an extract would depend on whether the tyrosinase or peroxidase systems were more active. Unpublished observations indicate that the two systems vary in activity independently during leaf development. Dialysis of the extract would depress the activity of both systems, in the case of tyrosinase by removal of the necessary polyphenols, and in the case of peroxidase by removal of H_2O_2 or sources of it. Each system could then be separately reactivated, by addition of polyphenols or H_2O_2 respectively.

An important uncertainty in this interpretation of auxin inactivation by crude extracts is that, although substrates for tyrosinase are undoubtedly present in such extracts (since they exhibit rapid browning), it is not known whether any of these phenols are active in promoting IAA destruction, as are catechol and pyrogallol. This question, as well as the possible physiological implications of the tyrosinase auxin destruction system, is currently under investigation.

TABLE II

INACTIVATION OF IAA BY DIALYZED EXTRACT OF DORMANT OSMUNDA FRONDS

ADDENDA *	TIME	AVENA CURVATURE	INACTIVATION
	hrs	*degrees*	*%*
None	9	28	...
Extract	0	28	
	6	26	8
Extract + catechol	0	24	
	3	9	68
	9	0	100

* Basal reaction mixture as in table I. Addenda: extract, 0.51 catecholase units per ml. Catechol, 10^{-3} M. Final volume 10 ml.

Summary

Indoleacetic acid is inactivated by mushroom tyrosinase or a tyrosinase present in dialyzed extracts of Osmunda leaves, but only when catechol or pyrogallol are also added. Tyrosine and DOPA do not promote IAA inactivation, although these phenols are oxidized by the enzyme. It appears probable that a reaction occurs between IAA and one of the intermediate compounds formed during the oxidation of catechol or pyrogallol. Possible mechanisms for the reaction, as well as its implications for auxin destruction in plant extracts are discussed.

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EFFECTS OF LIGHT ON THE RATE OF RESPIRATION IN THE STEM OF PISUM SATIVUM L.[1]

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It is well known that plants grown in the dark or under certain wave lengths of light produce stems of varying lengths and diameters. Kolda (2) showed that the difference in stem diameter of dark and light grown seedlings of *Pisum sativum* L. is brought about by variation in the growth of vascular tissues. Went (5) found that the result of exposure of peas (var. Little Marvel) to intermittent red and orange light for 6 min per day for 5 days was a 20 % to 30 % shortening of the stem compared to dark grown controls, while a single exposure of 30 min had no effect on the size of the stem; on the other hand, long exposure results in red-etiolation.

We have observed that continuous exposure of the upper parts of seedlings of *Pisum sativum* L. var. Alaska (42 to 96 hrs) to yellow, green, or red light results in the production of stems that are greater in length but slightly lesser in diameter than those grown for the same duration in natural daylight. Those exposed continuously to fluorescent daylight for the same duration produce stems that are shorter in length but greater in diameter than those grown in natural daylight.

The aim of this investigation is to determine if the growing of stems under varying light conditions affects the rate with which this tissue oxidizes certain salts of organic acids.

Pea seedlings were grown in metal flats containing vermiculite under natural daylight or in darkness. Others were grown in a specially constructed cabinet under continuous exposure to light emitted from 20-watt red, green, yellow or daylight fluorescent bulbs. The four bulbs were so arranged that two were on opposite sides at a distance of 10 cm from the edge of flats. All plants were harvested on the eighth day after planting.

Sections of tissue 4 mm in length were cut from the region immediately posterior to the youngest leaves.

Manometric measurements were carried out in calibrated Barcroft-Warburg reaction vessels at 30° C.

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TABLE I

ENDOGENOUS RESPIRATORY RATES OF STEM TISSUES OF PEAS GROWN UNDER DIFFERENT LIGHT CONDITIONS

LIGHT CONDITION	Q_{O_2}*	σ**
Natural daylight	3.10	0.14
Fluorescent daylight	3.93 ††	0.23
Green	3.10 †	0.16
Yellow	5.33 ††	0.32
Red	2.66 †	0.17
Darkness	3.90 ††	0.12

* Mean Q_{O_2} figures representing averages of 6 to 8 vessels of 3 separate experiments.
** Standard deviation of the mean.
† Significant to the 2% level of confidence.
†† Significant to the 5% level of confidence.

The shaking rate was 135/min through a radius whose arc was 4 cm. Five percent KOH with folded filter paper was added to the center well as the CO_2 absorbent.

All chemicals used were obtained from commercial sources and of reagent grade. Dry weights were obtained by heating the stem tissue to constant weight at 80° C.

The reaction system used for the oxidation of succinate was published by Umbreit, Burris, and Stauffer (4). The only modifications of their method were the use of 100 mg (wet weight) of tissue and the use of 0.02 M phthalate buffer of pH 4.5. The oxidation of citrate was determined by using 0.04 M sodium citrate, 0.02 M phthalate buffer of pH 4.5, distilled water to a total volume of 3.0 ml, and 100 mg (wet weight) of tissue slices. The same reaction system as for citrate was used in the determination of the oxidation of monosodium glutamate (0.059 M), glycerophosphate (0.05 M), and ascorbate (0.05 M), except for the substitution of each of these substrates for citrate in the respective determinations. The ascorbate was prepared immediately before use by neutralizing ascorbic acid with 0.1 *N* NaOH.

All data obtained from the experiments were treated for their statistical significance. The mean Q_{O_2} figures represent averages of results obtained from 6 to 8 respirometer flasks of three experiments after corrections for the endogenous Q_{O_2} (where Q_{O_2} is μl O_2/mg dry wt × hr). The standard deviation of the mean (σ) is included in the tabulated data. The mean Q_{O_2} values from plants grown in natural daylight were compared with those from other light conditions to determine the levels of significance of the data. As suggested by Fisher (1), we considered a probability of less than 0.05 to be significant.

Table I summarizes the results obtained from measurements of the residual respiration. As seen in the table, the endogenous respiratory rate of pea stem tissues grown under different light conditions was not constant. The rate of oxygen uptake by plants grown under red light was much lower than the others.

The results obtained from the oxidation of substrates by stem tissue grown under different light conditions are summarized in table II. The table indicates that the descending rate of oxidation of succinate was darkness, natural daylight, fluorescent daylight, green, yellow and red. The average oxygen uptake for succinate in dark grown seedlings was approximately 45% greater than those grown in the red light. The descending rate of citrate oxidation was green, yellow, darkness, fluorescent daylight, red, and natural daylight. The descending order of glutamate oxidation was darkness, red, green, natural daylight, and fluorescent daylight. Glutamate apparently was not oxidized by plants grown in the yellow. For

TABLE II

RESPIRATORY RATES ABOVE ENDOGENOUS VALUES OF STEM TISSUES OF PEAS GROWN UNDER VARIOUS LIGHT CONDITIONS

SUBSTRATE		LIGHT TREATMENT					
		NATURAL DAYLIGHT	FLUORESCENT DAYLIGHT	GREEN	YELLOW	RED	DARKNESS
Succinate	Q_{O_2}*	4.10	3.83 ††	3.76 ††	3.66 ‡	2.70 ‡‡	4.66 ‡
	σ**	0.10	0.12	0.12	0.28	0.10	0.17
Citrate	Q_{O_2}	1.73	2.80 ††	3.43 ††	3.36 ††	2.56 ‡	3.06 ††
	σ	0.14	0.10	0.34	0.23	0.31	0.10
Glutamate	Q_{O_2}	1.13	0.93 ††	1.43 ††	−0.13 ††	1.70 ††	1.96 †
	σ	0.07	0.17	0.39	0.14	0.21	0.03
Glycero-phosphate	Q_{O_2}	2.10	2.53 †	1.20 ‡	1.60 ††	2.60 ††	1.43 ††
	σ	0.07	0.26	0.20	0.14	0.17	0.21
Ascorbate	Q_{O_2}	29.50	29.86 ††	26.23 ††	26.36 ††	23.03 ††	23.60 ††
	σ	2.87	1.18	1.06	1.79	0.33	0.30

* Mean Q_{O_2} figures representing averages of 6 to 8 vessels of 3 separate experiments after corrections for endogenous Q_{O_2}.
** Standard deviation of the mean.
† Significant to the 1% level of confidence.
†† Significant to the 2% level of confidence.
‡ Significant to the 5% level of confidence.
‡‡ Not significant to the 5% level of confidence.

glycerophosphate, the highest value was obtained for red, followed by fluorescent daylight, natural daylight, yellow, darkness, and green. The descending mean Q_{O_2} values for ascorbate oxidation were fluorescent daylight, natural daylight, yellow, green, darkness, and red.

The lack of general conformity of the data in respect to the rate of oxidation of all the substrates by any one light condition indicates that broad conclusions are difficult to make. For example, stems grown in the darkness oxidize succinate more rapidly than tissues grown under other light conditions but ascorbate is oxidized more slowly by plants grown in the dark than by those in natural daylight. A similar case can be cited for yellow grown plants that oxidize succinate more rapidly than those of the red, but the latter oxidizes glycerophosphate more rapidly than the former. Ascorbate was oxidized at a much higher rate than any of the other substrates. There are several possible explanations for this. One is that several factors may be affecting the results. For example, cytochrome oxidase and ascorbic acid oxidase are both present in peas (Stafford, 3), and ascorbic acid can be oxidized by cytochrome c or ascorbic acid oxidase without the mediation of a dehydrogenase or coenzyme. In addition if polyphenol oxidase is present it can oxidize ascorbate in the presence of catalytic quantities of polyphenols.

It appears that citrate is not oxidized by pea stem tissues grown under yellow lights. The low values for citrate and glutamate are not explained by these experiments. However, if these substrates were to penetrate the cell membranes slowly, low Q_{O_2} values would result. Nevertheless, the low constant Q_{O_2} values obtained may be important in that these substrates could be slowly funneled through an oxidative cycle.

The slow rate of oxidation of citrate by stems exposed to natural daylight sheds some possible explanations on the effects of light on the rate of oxidation in plant tissues. One is that certain wave lengths of light may render the cell membrane more or less permeable to certain substrates. Conversely, the absence of these wave lengths might have a reverse effect. Such factors as optimum pH of the reacting system, concentration of coenzymes, and substrate-enzyme concentration would not account for these differences since all the experiments were carried out under similar conditions. Likewise these differences could not be accounted for on the supposition that light results in an increase in the nitrogen content of the tissue. If this were true, the quantity of enzymes would correspondingly increase with the other cellular proteins, resulting in higher Q_{O_2} values for all substrates oxidized by plants grown under the same given light conditions. For example, our observations indicate a more rapid oxidation of succinate by plants grown in darkness. If the lack of light causes an increase in enzymes of plant tissue, it would follow that citrate, glutamate, glycerophosphate, and ascorbate oxidation should be higher in plants grown in darkness than in those grown under the various light conditions mentioned. Such an idea indirectly gains some support from the findings of Kolda (2). This investigator found that light grown tissue contained more vascular tissue than those grown in darkness. Since a considerable portion of vascular tissue is non-living, then its absence or presence would not be significantly reflected in the oxygen uptake. Another possible role of light is that it probably causes greater accelerating effect on some enzyme systems than on others. This would tend to explain the variable nature of the data obtained for any given light condition.

Summary

1. Pea stems were grown under one or the other light conditions (darkness, natural daylight, or red, daylight, yellow, and green fluorescent bulbs) and studied with respect to their ability to oxidize succinate, citrate, glutamate, glycerophosphate, and ascorbate.

2. The descending order of oxidation of succinate was darkness, natural daylight, fluorescent daylight, green, yellow and red. The descending Q_{O_2} values for citrate were green, yellow, darkness, fluorescent daylight, red, and natural daylight. The highest rate for the oxidation of glutamate was obtained for stems grown in darkness, followed by red, green, natural daylight, and fluorescent daylight. No oxidation of this substrate was observed in the yellow grown plants. The descending rate of oxidation of glycerophosphate was red, fluorescent daylight, natural daylight, yellow, darkness, and green. The highest Q_{O_2} value for ascorbate oxidation was obtained for fluorescent daylight, followed by natural daylight, yellow, green, darkness, and red.

3. It is suggested that the differences that occurred in the rate of oxidation of the preceding substrates by pea stems grown under different light conditions were due to alterations of the cellular constituents produced by the presence or absence of certain wave lengths of light.

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AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS, *Appointments:* The following members have been appointed to serve on the Charles Reid Barnes Life Membership Committee: Hugh Gauch, Chairman, Orlin Biddulph, R. H. Burris, Paul J. Kramer and Jack Myers.

The Committee on Professional Status and Training under the direction of V. A. Greulach has appointed C. J. Lyon of Dartmouth to the vacancy caused by the resignation of K. V. Thimann.

M. A. Sprague has been continued as our representative on the Joint Committee on Grassland Farming. Hugh Gauch has been reappointed as our representative to the Agricultural Research Institute.

Our Society will once again meet with the American Institute of Biological Sciences (AIBS) next September. The meeting will occur at the University of Connecticut, Storrs, Connecticut. The local representative on the University of Connecticut campus is A. B. Schwarting, who will co-ordinate activities for our meeting.—ARTHUR W. GALSTON, Secretary.

PROTOPLASMATOLOGIA, HANDBUCH DER PROTOPLASMAFORSCHUNG, VOL. 2E—L. V. Heilbrunn and F. Weber, ed. Springer-Verlag, Molkerbastei 5, Wien 1. Part 4.—The Enzymology of the Cell Surface, A. Rothstein. 86 pages. Part 5.—Tension at the Cell Surface, E. N. Harvey. 30 pages. 1954. $6.80.

"Protoplasmatologia," to judge by its Table of Contents, is intended to be an exhaustive survey of the properties of protoplasm—physical, chemical, morphological and physiological. So diversified is the subject matter, and so uneven the range of topics ("The Gene," in one case, the "Contractile Vacuoles of Protozoa" in another) that one hesitates even to guess how effective a guide to the understanding of protoplasm the massive series will prove to be. Certainly, this "Handbuch der Protoplasmaforschung" will provide lots and lots of information. But, if the volume under review is any indication, one should not expect what one would most hope for—a balanced and integrated treatment of our present knowledge concerning what Huxley called the "stuff of life."

The subject of this volume is the cell surface. However, only two aspects of surface organization—enzymology and tension—are covered. The cytoplasmic cortex, the chemical nature of the plasma membrane of animal cells, and the plasma membrane of plant cells are assigned to other volumes. Tension at the cell surface is discussed by E. N. Harvey. He begins with a beautiful and interesting historical introduction on surface chemistry, but from this introduction one never quite emerges. The work of the past sixteen years is covered by some 20 references. There is an excellent discussion of problems encountered in the measurement of tensions at cell surfaces. But, if the discussion is good, the topic must be wrong, for the field of interest seems oppressively narrow. It should have been widened to include something about the physical nature of membrane organization drawing upon recent techniques of study such as those of electron microscopy. In this way tension phenomena might have been brought out of the past and into the framework of the present without being catapulted into the future. As it stands, the article is an isolated study entirely unrelated, except in name, to its companion topic of surface enzymology.

The contribution of Rothstein, if less scholarly, has a sweep which is most welcome. It is cautiously written and informative albeit a little tendentious. The author is obviously dedicated to the proposition that the cell surface is a seat of important enzymatic activities. His evidence is broadly drawn; his arguments, lucidly presented. His sense of biology is apparently strong enough to have kept his enthusiasm for generalizing in check. Only with respect to the active transport of solutes across cell membranes has temptation drawn his resistance thin. Although he favors, he does not insist, that the demonstration of an active transfer of sugars into yeast cells by surface enzymes is a model for all other cell types. Perhaps he should have emphasized that it needn't be. If, as he points out, the membrane is an important site of interaction between the cell and its evironment, then the environment in which a particular type of cell has evolved and for which it is adapted must have a significant bearing on the biochemical organization of its surface. Trillium microsporocytes which are bathed in a highly controlled and specialized environment (the sap of the anther) are freely permeable to mono- and disaccharides; yeast cells, living in an open and variable environment, are equipped with tightly controlled penetration mechanisms. But the important point, which Rothstein makes amply clear, is that in very many instances enzymes are incorporated into the organization of cell surfaces, and that such enzymes may function in the active transport of substances, in the synthesis of extracellular structures, and in the digestion of extracellular substances. The fourth function "self maintenance and perhaps replication of new surface during growth" is open to question. If the surface is to be designated as "self maintaining" then the issue is no longer one of biochemical mechanisms alone but also one of genetics. In the strict sense, no segment of a cell is self maintaining, a fact clearly recognized in the discussion. The term acquires significance only when the component in question has genetic continuity which requires it to govern its own formation. But has the surface of a cell genetic continuity? The question is intriguing but as yet without answer. Still, whatever the criticisms here raised, the article is a first rate discussion of enzymatic activities at cell surfaces and a stimulating contribution to our understanding of the physiology of the cell membrane.—H. STERN, Division of Chemistry, Science Service, Department of Agriculture, Ottawa, Ontario, Canada.